CONNECTIVE TISSUE
IN HEALTH AND DISEASE

EDITED

by

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INTRODUCTION

AFTER AGES OF RELATIVE INCONSPICUOUSNESS the connective tissue has been attracting increasing attention during the past 25 years, an attention that in the last decade has approached enthusiasm.

In 1928, Duran-Reynals discovered that a testicular extract was capable of increasing the permeability of connective tissue It was soon realized that this was due to an effect on the ground substance In the thirties, Karl Meyer and associates isolated a high-molecular, viscous polysaccharide from the vitreous body of the eye, a mucopolysaccharide which they named hyaluronic acid Subsequently, this mucopolysaccharide was found to be present in all connective tissue In combination with proteins it constitutes the mucinous component of the connective-tissue ground substance, of the articular fluid, of the ocular humours, of the jelly of the umbilical cord, and of the mucinous matrix of the granulosa cells of the ovum It was in Meyer's laboratory too that the enzyme hyaluronidase was discovered, an enzyme that was able to break down hyaluronic acid Shortly after, Duran-Reynals' testicular spreading factor was found to be identical with Meyer's hyaluronidase Hyaluronidase was demonstrated in the testes of mammals; it proved to be formed by invasive bacteria and by certain malignant tumours, to occur in the venom of snakes, leeches, spiders, and bees Blood and tissues contain substances capable of inactivating or inhibiting hyaluronidase, and the organism is able to produce a specific antibody against the antigenic enzyme

Another mucopolysaccharide contained in the connective-tissue ground substance, chondroitin sulphuric acid, possesses no particular viscosity and, unlike hyaluronic acid, only a slight capacity to bind water It is the most important component of the ground substance of cartilage

The fibrils are embedded in the ground substance During the past 10–12 years, the investigations into the structure of the fibrils have passed from the magnifications of the light microscope, by that of the electron microscope, to the molecular level Collagen and elastin are polypeptides, each with its characteristic content of amino acids. Electron microscopy,

X-ray diffraction, and chemical analysis have enabled us to distinguish between collagenous, reticular, and elastic fibres as regards structure, molecular orientation, and amino acid content. And we are able to observe changes caused by physiological factors, aging, etc.

Procollagen-carbohydrate complexes evidently precede the formation of fibrous collagen In some way or other glycoproteins appear to be essential to fibril formation.

It is now an established fact that connective-tissue cells are concerned with the formation of the intercellular substance. Fibroblasts appear to contribute to the formation of collagen fibrils, and the mast cells evidently produce components of the ground substance. All these facts lend particular importance to the recent studies performed on the morphology, chemistry, and physiology of the cells, perhaps mainly those concerning hormonal influence on the intercellular substance of the connective tissue

In the course of time, morphologists have learned that they need the aid of chemistry in identifying the constituents of the tissues Recent advances within histochemistry are utilized to the advantage of morphology as well as chemistry. Tinctorial histo-chemistry has its significant sources of error, but no chemical analysis—be it ever so accurate—is satisfactory, if structural details have to be located. Chemists are not very tolerant of inaccuracies on the part of histologists But imagination coupled with a common need and wish for progress has united the two parties; to-day chemists are at work in modern histological laboratories. Morphologists and chemists are partners in the same game And the established team-work has led to important results regarding the chemical morphology of the cells, of the ground substance, of collagen and elastic tissue, of loose mesenchymal structures, as well as of cartilage and bone.

Research into the structure and texture of the connective tissue has called upon the aid of physicists They have joined the chemists in the application of radioactive isotopes Histological sections are now submitted to autoradiography Histospectrophotometry is another field of immense significance And the morphologist must have insight into physics in order to master electron microscopy, X-ray diffraction, freeze-drying of tissues, etc.

Physiological experiments show the variability of the chemical and physical structure and state of the connective tissues. The metabolism of the intercellular substances is an object of intensive research because of its basic relevance to mesenchymal pathophysiology Vitamin C has appeared

on the scene; it has proved to be a factor of some importance in the formation of mucopolysaccharides and collagen fibrils Physiology completes the bridge to pathology and medicine.

Pathologists have to tackle the problems of the pathological tissue changes and reactions occurring in connective-tissue disorders. Gradually, some knowledge has accumulated about fibrinoid, hyaline, amyloid, and paramyloid through chemical analyses, histochemical staining reactions, and histophysiological experiments The presence of these changes in the intercellular substances in connective-tissue diseases, of amyloid in scurvy etc has set us thinking The reticulo-endothelial system participates in some connective-tissue reactions in a way that still remains to be elucidated Pathogenetic considerations regarding mesenchymal diseases are not lacking in number, but we are still without positive and definite conclusions.

In 1949, Hench and associates published their sensational discovery that the adrenocortical hormone, cortisone, has a dramatic effect on acute and chronic arthritis This report was followed by a multitude of publications on the beneficial clinical effect of this hormone on diseases of the skin, eyes, blood vessels, and other organs—diseases of connective tissue wherever it occurs

When it was realized that cortisone and the adrenocorticotrophic hormone of the hypophysis, ACTH, acted primarily on the mesenchymal tissues, renewed and extended interest in these tissues was awakened There was a general feeling that by following these results up, there might be a chance of relieving much human suffering and alleviating great social-economic burdens.

The first clinical reports were soon followed by publications from laboratories all over the world on the morphological alterations caused by cortisone in mast cells, fibroblasts, and intercellular substance, on chemical and physical changes, on changes in the turnover of the tissues The effects of cortisone and ACTH upon wound healing, infection, and tumour development were studied. Parallel studies were performed on the actions of hydrocortisone, desoxycorticosterone, and other adrenal steroids It became evident that the thyrotrophic hormone and the growth hormone of the hypophysis exerted effects directly on the connective tissue that seemed exactly opposed to those of cortisone. Cortisone inhibited connective-tissue formation, whereas thyrotrophin and growth hormone stimulated it with a slight and a strong stimulating action respectively on fibril formation.

And thyroxin inhibits all the actions of thyrotrophin—its fat-mobilizing, its exophthalmogenic as well as its thyroid-stimulating effect.

Gradually, experience could again be carried over from the laboratories to clinical practice It yielded an acceptable explanation of the effect of thyroxin upon myxoedema, the effect of cortisone upon rheumatoid arthritis, etc. Hormone therapy was introduced in diseases without any signs of hormonal insufficiency, but exhibiting tissue changes promising a beneficial influence of hormones. For example, the accumulation of hyaluronic acid in disseminated lupus erythematosus could be expected to be amenable to cortisone The same applies to rheumatoid arthritis, rheumatic fever, pemphigus, scleroderma, dermatomyositis, periarteritis nodosa, and a number of other conditions

To-day, a collaboration between the clinicians and their colleagues in the laboratories is a matter of course Laboratories are built in conjunction with *clinical departments. Clinicians are at work in the laboratories* Large sums are expended to procure the most modern facilities for research work.

Joint diseases have perhaps received more interest in the medical world than any other connective-tissue disorders in the past five years. The effect of cortisone upon the articular connective tissue, upon the synovial mucin and its formation plays an outstanding rôle. Similarly, the aspects and problems of inflammation and degeneration, tissue metabolism, the visco sity and chemistry of the lubricant are still in the focus of interest.

Dermàtology embraces an important group of connective-tissue diseases. Most skin diseases involve connective tissue, and interest has therefore primarily centred on the systemic disorders that come within this field. The past five years have revolutionized dermatological diagnosis and therapy, and this external medicine has joined in the common struggle to solve the mystery of the pathological reactions of the connective-tissue system.

Ophthalmology also has its connective-tissue problems, particularly as regards vascular diseases as well as changes in the iris and uvea. The ocular humours originate from the mesenchyme They appear to alter under hormonal influence just like the articular fluid. It is not yet clear, whether the capacity of the hyaluronic acid of the humours to bind water is a factor in regulating the intraocular pressure. Exophthalmos appears to be due to changes in the retrobulbar connective tissue, provoked by hormonal dysfunction.

Connective tissue plays an important rôle in the development and

growth of benign and malignant tumours, not only mesenchymal but carcinomas as well. Mast cells and tissue polysaccharides are predominating elements in their connective tissue Hormones may inhibit or stimulate the growth and spread of tumours; they may even bring fully developed tumours to regression.

Arteriosclerosis and other cardio-vascular diseases start in the mesenchyme of the vessel walls An increase and a change of the ground substance of the intima and media may be observed at an early stage of the disease process Subsequent phenomena are fibrosis, deposition of lipids, and calcification

Wound healing is primarily a new formation of connective tissue Inhibition of the formation of ground substance interferes with wound healing The process is subject to inhibiting and stimulating hormones. The healing of bone fractures, the formation of pleural and peritoneal adhesions following operations, and other traumatic fibroses are governed by similar factors

The severity of bacterial and viral infection depends on the condition of the connective tissue at the moment concerned, its spreading in the tissues depending on the permeability of the ground substance and on the hormonal state which on the other hand may be influenced by various external factors. The hyaluronidase of the bacteria may find a more or less ideal substrate And in the moment of degradation the break-down products of hyaluronic acid will reject the attack, bringing an inhibitory effect to bear upon the enzyme and even upon the aggressor Resistance against infection is largely a matter of connective tissue

The hyaluronate binding the granulosa cells of the ovum together and the hyaluronidase of the spermatozoa are controlled by endocrine regulators Thus, even conception is dependent on connective-tissue physiology

The responses of mesenchyme to various external and internal actions are identical, whether we are dealing with joints, skin, eyes, vessel walls, bone-marrow, tumours, granulation tissue or inflamed tissue To-day rheumatologists do not hesitate to study connective-tissue elements in a cancer, if it is more easily accessible than a joint And dermatologists may advantageously study the chemistry of the synovia.

Connective tissue connects the numerous branches of medical science. Without connective tissue, medicine would come to pieces, even non-viable pieces, just like the cells of the human body.

The contributors to this book include the pioneers of modern connec-

tive-tissue research. Their names are known to everyone concerned with connective-tissue problems.

The contributions have been made in the hope to acquaint the medical world with the status and significance of connective-tissue science by means of surveys bringing each aspect up to date

This volume is intended to present the connective tissue in the strict sense of the word Our primary aim was to bring the variations in the elements of normal connective tissue to light The book should be accessible to all members of the medical profession with the most varied qualifications and interests The subjects treated might have been more numerous, and the list of contributors ought to have been longer. However, on behalf of all the contributors, I ask our readers to receive the publication with tolerance of its shortcomings and with sympathy for its aim.

In the list of contributors, we miss the name of one of the most eminent scientists within recent connective-tissue research, Dr. Henry Bunting of the Yale University, U S.A A few months ago, he joined our work with enthusiasm The chapter on "Aging of Connective Tissue" was to have been written by him in collaboration with Dr. Banfield; but he died before he had started, after a short illness, 42 years old. His death was a tragedy to us who knew him and a serious loss to his branch of science.

G. Asboe-Hansen

NORMAL MORPHOLOGY AND MORPHOGENESIS OF CONNECTIVE TISSUE

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Historical Introduction

"It is sufficient to know, that all the Parts of the Body are made up of Threads, or Fibres, of which there be different Kinds, for there are some soft, flexible, and a little elastick, and these are either hollow, like small Pipes, or spongeous, and full of little Cells, as the nervous and fleshy Fibres; others there are more solid and flexible, but with a strong Elasticity or Spring, as the membranous and cartilaginous Fibres; and a third Sort are hard and inflexible, as the Fibres of the Bones. Now of all these, some are very sensible, and others are destitute of all Sense, some so very small as not to be easily perceived, and others, on the contrary, so big as to be plainly seen. And most of them, when examined with a Microscope, appear to be composed of still smaller Fibres.

Now these Fibres do first constitute the Substance of the Bones, Cartilages, Ligaments, Membranes, Nerves, Veins, Arteries and Muscles. And again, by the various Texture, and different Combination of some or all these Parts, the more compound Organs are framed, such as the Lungs, Stomach, Liver, Legs and Arms, the Sum of all which make up the Body."

This paragraph, with slight modifications, would provide a passable summary of the modern viewpoint on connective tissue, although it is taken from James Keill's "The Anatomy of the Human Body, abridged", which was first published in 1698 and was one of the most popular anatomy books for English students for nearly a century.

Berg (1942) has made an excellent study of the historical evolution of the fibre concept, showing that it can be divided into a number of

stages. Until the Renaissance the idea of a fibre structure was restricted to those tissues such as tendons and muscles which clearly have a fibrillary pattern; Fernel put forward the idea that all organs and tissues were built of a network of fibres and this was expanded so that the fibre pattern became the basis of a mechanistic pathology. It was natural that Bichat, when setting out his fundamental idea that there were a limited number of tissues from which the organs were formed, distinguished a fibrous tissue and consequent on this the concept of connective tissue developed, the actual term ("Bindegewebe") having been introduced by Johann Muller in 1830.

Virchow and Connective Tissue

It is not widely appreciated that Virchow's views on cellular pathology evolved from his study of connective tissue, which he regarded as intercellular substances of varying chemical nature in which cells were embedded and that the cells of connective tissue were analogous to those of cartilage and bone. Nevertheless he did not believe that the intercellular substance was derived from the cells; it was a mere packing material and the cells were the significant portion. However, with the acceptance of the cell theory, came the views of Max Schultze that the fibres and intercellular substance of connective tissue were extensions and elongations of the cytoplasmic protoplasm into a symplasm; this tended to discourage the furtherance of Virchow's views of the unity of the various forms of connective tissue cell and his hypothesis lost favour still more when von Recklinghausen showed that in inflammation, the pus cells were probably derived from the leucocytes of the blood stream rather than locally from connective tissue cells, as Virchow had believed.

With the emergence of the concept of cellular activity and dominance in biological reactivity, the functional significance of connective tissue was lost sight of and the idea has only reappeared in the last decade. This loss of interest in connective tissue was not so much due to lack of knowledge as to scientific isolationism. The development of histology and biochemistry resulted in a static morphological approach on the one hand, and an interest in general metabolism or the isolated chemical constituents of the cell on the other hand, so that the morphological chemical approach that Virchow attempted was forgotten. As late as 1899, Sir William Hardy forsook the field of histology as he believed that advances in our

knowledge of the structure of tissues must await advances of our knowledge of the molecules which composed them and his contribution was the development of colloid chemistry

The Connective Tissue Renaissance

Once again it has been the climate of thought rather than new knowledge which has fostered the resurgence of the concept of spacial biochemistry; the fact that a morphologist can think, as well as speak, in terms of chemical reactivity and that a chemist can understand the significance of cellular structure in relation to metabolic function, opens up fields of endeavour as enthralling as those presented to the physicist and the chemist in the 1890's

The renewed interest in connective tissue has stemmed from a number of sources Leather chemists had for years been studying the structure of the dermis and the factors involved in tanning and it was fortunate that there were working in these industrial laboratories, men of vision and knowledge, such as Astbury & Highberger, who laid the foundations of molecular biology, however, in the early days their morphological studies were in the field of crystallography and X-ray diffraction analysis, which, for their understanding, demanded a far greater knowledge of physics than the average biologist possessed The bridge was provided by the electron microscope, for its micrographs could be appreciated by the histologists and the amplifications of its findings were able to confirm the deductions of molecular biology, though, as Frey Wyssling somewhat wistfully remarks, as a concequence, sub-microscopic morphology has lost something of its mysterious charm Nevertheless, the stimulus and integration of isolated scientific disciplines that this new technique has induced is as spectacular as was achieved when Amici and Lister perfected the achromatic objective that enabled Schleiden and Schwann to display the science of cytology. The other source has been from pathology In 1933, Klinge, as a result of comparing the lesions in rheumatic fever with those induced in animals receiving repeated protein injections, concluded that the essential pathogenesis of rheumatic fever was an alteration of the mesenchymal ground substance and collagen fibres induced by bacterial hyperergy Nine years later, Klemperer, Pollack & Baehr's classic paper introducing the concept of Diffuse Collagen Disease appeared and created little attention It was not until 1949, with the introduction of cortisone and its apparent

efficacy in the treatment of a number of disorders involving connective tissue, that the concept of collagen diseases received what Klemperer so rightly calls "an exaggerated popularity" Nevertheless, the clinical stimulus to research in the field of connective tissue has only been beneficial and the advances in our understanding of its morphology and morphogenesis in five years has been quite remarkable.

The Character of Connective Tissue

Connective tissue may be regarded as a continuous fluid matrix varying in consistency from the limpid Wharton's Jelly of the umbilical cord to the hardness of bone, in which is lying an interlacing fabric of fibres of different sorts and is bathing cells which may be isolated or closely set, orientated to form an integumentary covering, tubules for absorption, secretion or excretion, the transmission of oxygen, blood or lymph or specially patterned as muscles or nervous tissue. Naturally the cells cannot be ignored; Virchow's dictum may have been over-narrow, yet it is the cell and its activity which ultimately determines the pattern of the extracellular matrix

The character and thickness of the connective tissue layer varies from organ to organ In the lungs and endocrine glands it is extremely thin except for the capsule; in the liver and kidneys it is somewhat thicker and in the heart and muscular organs it forms a significant portion of the viscus; this is also true of the intestinal tract and the skin, while the bones, joints and tendons are formed entirely of connective tissue, but it is important to remember that the connective tissue is continuous throughout the body.

It is really immaterial which organ is selected to study the connective tissue in greater detail, but the dermis gives an excellent range of pattern, though it will be necessary to discuss certain aspects of other organs.

Connective Tissue in Skin

A section of skin examined at moderate magnification reveals the relationship of the epidermis and its appendages to the connective tissue dermis in which are lying blood vessels and nerves as well as the sweat and sebaceous glands. However, it is well to remember that a thin section is liable to be misleading in some respects; for instance, the blood supply appears very sparse whereas a thick injected section of the same sort of

region reveals the rich blood supply that there is in connective tissue A section stained by haematoxylin-eosin reveals that the dermic connective tissue consists of an admixture of cells and fibres and structurally three main zones can be made out which merge imperceptibly one with another and correspond well with the main varieties of connective tissue. The stratum papillare is just deep to the dermis; it appears almost homogeneous and is rich in cells. Deep to this is the stratum compactum or reticulare in which there is a close set network of interlacing bundles of fibres which are relatively acellular and the majority of the cells are orientated in relation to these fibrillary bundles. The next layer, the subcutis, is rich in fat spaces interlaced by loose connective tissue

THE CELLS OF CONNECTIVE TISSUE

The cells which can be identified in normal connective tissue are the elongated fibroblasts, lymphocytes, plasma cells, and perhaps histiocytes and neutrophil leucocytes, while fat cells will be present in the subcutis. Tissue mast cells or basophils may not be recognised or mistaken for plasma cells with ordinary stains, for the cytoplasm is acidophil, although the cytoplasmic outline is not so precise, however, when a section is stained with a metachromatic dye, the large number of mast cells in connective tissue is often surprising Similarly the histiocytes and undifferentiated mesenchymal cells (or reticulum cells) are difficult to recognise, the former being confused with fibrocytes and the latter can only be inferred from their nuclear character and perivascular situation, their extent is revealed by a silver impregnation method of the Hortega microglia type (Marshall 1953)

THE FIBRES OF CONNECTIVE TISSUE—COLLAGEN AND ELASTICA

Just as it is necessary to use special stains to reveal the cytology of connective tissue, so special techniques are required for the extracellular components The dermis examined under polarized light reveals an interlacing fabric of birefringent bundles in the stratum compactum; these are collagen, the principal fibrillary component of connective tissue, and the reaction under polarized light indicates that these bundles are made up of orientated fibrils They have characteristic staining reactions, staining red with Van Gieson's picrofuchsin and having a strong affinity for acid aniline dyes when subjected to the Mallory or Masson Trichrome process By using any of these methods, collagen fibres, in addition to the dense bundles

in the stratum compactum, will be found in a loose arrangement in the stratum papillare or in strands in the cutis On the other hand, if a section of skin is stained with Weigert's resorcin-fuchsin or orcein, the collagen fibres will not be stained, but fine branching fibres of elastic tissue, another of the fibrillary components of connective tissue, will be apparent, predominantly in the stratum papillare but also running with the collagen bundles in the stratum compactum and with the collagen strands in the cutis

RETICULIN, BASEMENT MEMBRANE AND GROUND SUBSTANCE

If the dermis is examined under higher magnification, when impregnated with silver by one of the modifications of the methods originally described by Maresch and Bielchowsky, it will be seen that the interlacing pattern of dense bundles of collagen which were birefringent under polarized light stain a light golden brown and are not so conspicuous as finer black fibrils, so-called reticulin, which are principally in the stratum papillare, and it can be seen that these are condensed at the dermic epidermic junction and around the blood vessels—the basement membrane; furthermore there appears to be an imperceptible merging both structurally and tinctorially from the fine black branching reticulin fibrils to the coarser golden brown bundles of collagen If the section of skin is treated with periodic acid followed by Schiff's reagent—a method known as the Hotchkiss or McManus stain, which, with certain limitations and provisos which need not be detailed here, will reveal the presence of carbohydrate—it will be seen that the spaces between the collagen fibres stain faintly, while around the reticulin fibrils it will stain a more intense red. So we are beginning to get an idea of the nature of connective tissue—it consists of an interlacing network of collagen, reticulin and elastica fibres lying in a non-fibrillary matrix of ground substance which has a variable carbohydrate content; scattered through the tissue are a variety of isolated cells.

It will be necessary to consider the cells of connective tissue when its morphogenesis is discussed, but it would probably be better to start by examining the extra-cellular components of which the principal one is collagen.

Collagen

What is collagen? It is well to remember Whitehead's dictum "that the scientist, like Humpty Dumpty, enjoys the privilege of paying words extra

and making them mean what he likes", a group of distinguished scientists spent a considerable portion of a meeting on connective tissue trying to agree on a definition acceptable to all of them and in the end returned to etymology—that it was glue or gelatin forming stuff!

The difficulty lies in the fact that it is not at all easy to compare directly specimens submitted to various methods of investigation, and so it is impossible to be sure, for example, that material which reveals a particular type of X-ray diffraction pattern is in fact a sample of collagen as the histologist understands it.

Histological and Chemical Characters

To the histologist collagen means bundles of fibres of varying thickness, which, when fixed, stain in characteristic manners; when unfixed the fibres swell and may to some extent dissolve when treated with weak acids, they resist tryptic digestion, but are digested by pepsin and by filtrates from cultures of certain bacteria. To the chemist collagen is a protein with rather remarkable properties, in acid solutions it swells more than any other protein, it is insoluble in water, but on heating is converted to a water-soluble substance—gelatine. The amino acids of the polypeptide chains are also unusual, a third of the residues consist of the amino acids proline and hydroxyproline, and a third of glycine together with a small amount of carbohydrate.

Physical Characters

The physical characters of collagen are also of interest. In large bundles it has practically no extensibility but if heated to a temperature of about 60° C it contracts to about a quarter of its original length and then develops a rubbery long range elasticity, that is to say, it can be stretched to its full length but flies back when released. On cooling the fibres almost return to their natural length but retain some degree of elasticity which the normal non-heated fibre does not show.

Ultrastructure of Collagen

The configuration of the polypeptide chains in collagen have been deduced with the aid of X-ray diffraction and infra-red dichroism and various models of the molecule—some sheet-like, some cylindrical or spiral, have been propounded but none, so far, explain all the known facts.

Under the electron microscope collagen fibres show a cross-striated appearance with a periodicity of 640 Å, subsidiary bands can be made out on occasions and it is suggested that the basic repeating unit is a 210 Å period and that three of these combine to form the ordinary prominent period There are many unresolved problems in relation to the submicroscopic morphology of collagen, such as the mode of lateral alignment of the fibrils, the fact that individual fibrils can be extended ten-fold even though collagen fibres are virtually inextensible and whether the individual fibrils are hollow rods or not

THE FAMILY OF COLLAGENS

So far we have spoken of collagen and by inference have been dealing with mammalian material, yet when we consider the matter from the point of view of comparative anatomy we must talk of "collagens", and as is so often the case in the broad biological fields, we began to wonder how large the collagen family should be. If we take mammalian fibrous tissue as a "typical collagen", though the justification for this might be questioned by members of other phyla, the problem of mammalian reticulin is immediately before us and will be discussed a little later on. The collagen of birds' tendons conforms closely to mammalian collagen, but in fishes both ichthyocol and elastoidin lack certain chemical and structural features and these differences become gradually more marked as we move down the animal kingdom to the spongin of the porifora; that is not all, for there is a completely distinct group of "collagens" which are classed as secreted in that the majority of them are derived from epithelial cells or glands rather than connective tissue They range from the ovo-keratin of the skate egg capsule, the byssus threads of molluscs, to the cuticles of worms, and though they do not give the cross-striated appearance on electron microscopy or the large scale structure that can be arrived at from low angle X-ray studies, yet they do have the characteristic collagen type high angle X-ray diffraction pattern and so to some extent might be regarded as incomplete collagens analogous to gelatin, a degraded collagen.

Even in mammalian collagens, which show little variability in chemical constitution or ultrastructure, there are striking physical differences. It has been known for many years that if rat tail tendon is placed in a weak solution of acetic acid, it swells and ultimately appears to go into solutions of high viscosity which can be filtered through gauze; if salts are added

to the collagen suspensions or there is an alteration in pH, collagen fibres with the 640 Å period are precipitated; however, these collagen "solutions" can only be readily prepared from the collagen of rat tail tendon; other rat collagen is relatively "insoluble" and the degree of "solubility" of human collagen, varies according to the site and age of the individual from whom the specimen is obtained In the light of these rather crude physical variabilities in material which is histologically and clinically identical, it is hardly surprising that there is considerable evidence of functional variability in collagen occurring in different sites in the body

PROCOLLAGEN

Another aspect of work on the nature of collagen has arisen from studies of the material first isolated by a group of Russian scientists and called by them procollagen, it is obtained by dialysis of a citric acid extract of skin, and on examining this material with the electron microscope, it is found to contain a mixture of the normal 640 Å collagen fibres and fibres with a very long-spacing 2,000–3,000 Å. If this procollagen is purified, no fibrillary collagen is formed, and it occurred to Highberger, Gross & Schmitt (1951) that one stage of the purification might remove glycoprotein; they added glycoprotein to purified collagen, and according to the concentration and pH, they produced varying proportions of long-spacing or normal-spacing collagen fibres; on the other hand polysaccharides, such as hyaluronic acid or heparin or chondroitin sulphate, did not induce fibre formation and they concluded that a carbohydrate fraction was essential for fibre formation and that certain complex chemical substances of a non-specific nature had to be present to induce precipitation and alignment of the protofibrils. Schmitt and his colleagues have recently described a third form of particle, a polarized asymmetrical type of long-spacing segment, which can be precipitated from collagen "solutions" by the action of adenosine triphosphoric acid, further they have shown that the three structural forms of collagen are interconvertible under suitable conditions.

Reticulin

The nature of reticulin and its relationship to collagen has been a matter for discussion for the last fifty years or so and the problem has been to obtain samples of reticulin free from collagen. I believe that Harold

Kramer (1953), working in my laboratory, has cleared away much of the confusion by examining with the electron microscope, X-ray diffraction, etc., material which histologically consisted of reticulin almost free from histological collagen It would seem that reticulin consists of a network of very fine disorientated fibrils of about 100 Å in diameter but showing the 640 Å periodicity of collagen; this network is lying in a membrane which appears to consist of polysaccharide and non-fibrillary collagen, probably only partially orientated; it has been suggested that the argyrophilia is due to the presence of mucopolysaccharide and certainly recent chemical analyses would suggest that reticulin has a much higher content of carbohydrate than collagen.

Elastica

The nature of elastica is at the present time somewhat obscure. Chemically it is a polypeptide with a high content of glycine, alanine and valine but a very low content of polar residues and X-ray diffraction would suggest that the molecules are largely disorientated, as is the case with rubber, which would be in keeping with its elastic properties. However, stretched elastic fibres show positive areas of refringence indicating the presence of orientated long chains and this alternation between disorientated and orientated long chain molecules is typical of structural proteins showing true elasticity.

The earliest electron micrographs were made by Wolpers who found branching fibres lying in a membrane, then Gross (1949) prepared elastica and found it to consist of fine twisted threads lying in an amorphous matrix, but subsequently he and others found that these threads were probably artefacts derived from the trypsin solution and were not elastica at all. Recently Hall, Reed & Tunbridge (1953) have described elastica as made up of two components—ill-defined, branching threads lying in an amorphous matrix, the proportion of the two elements differing from one site to another with a variable content of sulphate polysaccharide in combination with the protein, the non-fibrillary material having the higher polysaccharide content. Lansing (1952) has obtained similar results with the electron microscope, but much work remains to be done with elastic fibres and a profitable approach is likely to be with the aid of the pancreatic enzyme "elastase" (Banga & Balo, 1953).

Ground Substance

Lastly it is necessary to consider the ground substance, which in part consists of tissue fluid, derived largely from the blood plasma, but in addition it has a high content of mucopolysaccharides and glycoproteins (which are mucopolysaccharide-protein complexes) in a varying degree of polymerization, ordinarily it has a gel-like consistency and the histological evidence would suggest that the glycoproteins are most highly polymerized and therefore less soluble at the basement membrane where it is in close relationship to the reticulin and collagen fibres, but in the inter-fibrillary spaces it is less condensed and may, under abnormal conditions, become depolymerized, more fluid and finally water soluble

Under the microscope the ground substance appears optically homogenous, indeed its very existence was questioned for many years Its chemical constitution can only be determined by rather fierce extraction methods, while the histo-chemical methods available are also relatively crude, the Hotchkiss or Periodic Acid Schiff reaction merely indicating the probable presence of polysaccharides, and under certain abnormal conditions ground substance may show the phenomenon of metachromacy, that is to say the development of a pink colour on the application of certain dyes such as toluidine blue, which, with reservations, indicates the presence of high molecular weight sulphated mucopolysaccharides such as chondroitin sulphate or heparin, the non-acetylated sulphuric ester (Persson 1953) Last year Kramer & Windrum (1954) devised a method of studying the metachromacy of tissues before and after esterification and this will certainly widen our understanding of ground substance, it is already clear that there is a far greater range of polysaccharides present in connective tissue than have as yet been extracted chemically The enzyme hyaluronidase—the spreading factor of Duran-Reynals—which hydrolyses both hyaluronic acid and chondroitin sulphate has also been used to study changes in ground substance, but like other enzymal analytical methods, it has considerable limitations

Varieties of Connective Tissue

To recapitulate, connective tissue can be defined as *tissue fluid in a* matrix of mucopolysaccharides and scleroproteins, in varying degrees of polymerization but largely disorientated, in which are lying orientated fibres of varying thickness of collagen and elastica There are structural variants

ranging from the limiting and basement membranes which consist of a thin membrane of polymerized mucoprotein in which fine collagen fibrils are coursing to tendon where there is a high proportion of coarse collagen fibre with a variable admixture of elastica, embedded in a mucopolysaccharide matrix.

Cartilage

In the formation of cartilage there is an increased metachromacy of the ground substance indicating a higher content of chondroitin sulphate, while the coarse collagen fibres become more widely separated, and the mesenchymal cells divide, become swollen, with a high cytoplasmic glycogen content and then become grouped but widely separated in the highly polymerized ground substance which is cartilage. It is rich in orientated argyrophil fibrils resembling reticulin except that they show little branching but their patterning is significant in relation to the shaping of cartilage. In addition there are collagen and elastic fibres and in fibro and elastic cartilage these are in higher proportion. Precise chemical analysis of cartilage is lacking, it contains about 70 per cent water and of the dried residue, 80 per cent consists of collagen and chondroitin sulphate in varying proportions according to the site from which it is obtained; there is good evidence that much of the mucopolysaccharide is combined with collagen in the form of glycoproteins. Martin (1953) has recently described the presence of large numbers of fine non-striated fibres in cartilage which have different chemical reactions from collagen although they may be a collagen precursor.

Joints

A joint is merely an area of connective tissue free from cells and fibres in which the ground substance—in the form of synovial fluid—consists of tissue fluids with a high content of hyaluronic acid Edlund (1949), using the manometric method evolved by McMaster, has shown that the tissue resistance of a joint cavity is identical with that of loose connective tissue and in the formation of bursae and ganglia we have the development of miniature joints in connective tissue

Bone

The formation of bone is essentially a patterned calcification of ground substance associated with increased vascularisation, and although as a

histologist one distinguishes bone formation in fibre, by apposition and enchondral ossification yet the changes in all three are really the same; there is an increase of mucopolysaccharides in the ground substance with a separation of the collagen fibres and a morphological modification of the connective tissue cells, there is a change to an alkaline pH with depolymerization of mucopolysaccharide and then perhaps a phosphorylative glycogenolysis enzyme cycle proceeds in which the alkaline phosphatase may act as a transphosphorylase and the glycogen is derived from cellular constituents whether fibrocyte or chondrocyte The resultant is the formation of a phosphate calcium acceptor in the ground substance or cartilaginous matrix and calcification commences There is much dispute as to the stages that take place in calcification but it is generally believed that bicalcium phosphate is first formed and then is transformed to tricalcium phosphate, calcium carbonate and apatites The ultimate formation of bone is determined by the patterned arrangement of collagen and elastic fibres in relation to the bone salt crystals which are about 400 Å and 25–50 Å wide and lie between the collagen bundles (Rouiller et al 1952, Robinson & Watson, 1952)

Morphogenesis of Connective Tissue

Although there is considerable information about the nature of the constituents of connective tissue, their morphogenesis is far less understood It is clear that under ordinary circumstances the connective tissue cells known as fibroblasts are the cytogenic foci and originally it was believed that fibrin fibres, as extensions of the fibroblast cytoplasm, were converted into collagen but this now can be definitely excluded, although it may well be that when connective tissue fibres are forming, fibrin, by contraction, plays a part in the orientation and alignment in a purely mechanical way (Buck, 1953). However, embryonic mesenchyme is rich in mucopolysaccharides but is relatively acellular and in this collagen-type fibres appear, while Doljanski & Roulet (1933) showed, in tissue culture, that the formation of collagen fibres could occur in a portion of a fibroblast culture isolated from the cells, and they suggested that the fibroblasts secreted an enzyme, which acted on a substrate, contained in the plasma and embryonic extract of the culture medium. More recently Keith Porter (1951) has studied tissue cultures of fibroblasts with the electron microscope and has found that although cross banded collagen fibres are formed, yet these

often have a different periodicity from mature collagen fibres, some having a period of 210 Å and occasionally fibres with a spacing of 100 Å are observed, the fibroblasts appear to be surrounded by a fringe of short collagen fibres and Jackson (1954) has described intracytoplasmic filaments which appear to become continuous with the fibrous stroma. It is known that in embryonic or growing connective tissue there is often a high proportion of short collagen fibres with tapered ends and these may have a cross striated period of 210 Å instead of the 640 Å seen in mature collagen.

From metabolic studies it would appear that in the initial stage of fibrogenesis there is the formation of a non-fibrous soluble protein, analogous to the Russians' procollagen, and gradually the soluble procollagen-carbohydrate complex becomes dissociated during conversion into the insoluble mature collagen fibre. In fibroblast tissue-cultures, Mancini & de Lustig (1950) have found that hyaluronidase, which hydrolyses certain mucopolysaccharides, inhibits the formation of collagen fibres.

Accordingly it would seem likely that mucopolysaccharides play an essential part in collagen fibrillogenesis and that the fibroblast at least potentiates this and may be associated with the protein synthesis, but it is clear that other factors are also involved. For instance, in the healing of wounds in scurvy there is a deficiency of collagen and reticulin formation and the ground substance contains a gelatinous fluid which is not metachromatic but has a high protein content; the fibroblasts have the cytological characters associated with active formation of cytoplasmic protein and there is an increase of glycoprotein in the serum. On exhibiting ascorbic acid, the ground substance shows metachromasia, there is formation of reticulin and collagen, and serum glycoproteins fall. It would be reasonable to infer that ascorbic acid assists in the polymerization of mucopolysaccharides and that this is necessary for collagen fibril formation, although it may be that the scorbutic fibroblasts are only able to form a globular rather than a fibrillary scleroprotein of the collagen type; on the basis of Fell & Danielli's (1943) experiments, it had been suggested that phosphatase was essential for connective tissue fibre formation, but recent work would make it more likely that the phosphatase is adsorbed on to the fibroblasts from the epidermis (French & Benditt, 1954). Kramer (1952) has put forward the hypothesis that mucopolysaccharides might act as anionic detergents and by breaking down linkages in the globular form of collagen, which has been synthesized through the medium of the fibroblast, would promote curling and some degree of orientation of the collagen

molecules, and that the collagen protofibrils crystallise as the carbohydrate moiety is lost by dissociation, due to a change in the local electrolyte concentration or enzyme activity This hypothesis is in keeping with the facts of fibrillogenesis as far as they are known, conforms to known processes of synthetic fibre formation, and would explain the formation of collagen in sites relatively isolated from cellular activity as is often seen in pathological changes It is of some interest that Mercer (1952) has put forward an analogous mechanism for the biosynthesis of silk fibroin.

Even though information about collagen morphogenesis is scanty, it is lavish compared to that available for elastica and the ground substance. In the embryo and in healing wounds elastica appears much later than the collagen fibrils, and indeed it would appear that collagen is a prerequisite for its formation It is generally agreed that it is formed extracellularly and it seems that in an area of elastica formation, there is usually an excess of metachromatic material which takes on a beaded form and then fine non-agyrophil metachromatic fibres appear which gradually develop the staining reactions of elastica, at first the fibres are irregularly arranged in a fine meshwork and then develop into the fine branching and coiled refractile threads which are characteristic of elastica Hass (1939) has suggested, on the basis of some interesting experiments, that it might be formed as a fibrillary membrane at lipoid aqueous interspaces in the tissues and such a mechanism would be in keeping with observations such as those of Montgomery (1943/4) on the healing of experimental wound of the lung

There are two schools of thought with regard to the source of the mucopolysaccharides of the ground substance The presence in fibroblasts of granules and vacuoles having the histochemical reactions of mucopolysaccharides and the occurrence of large amounts of mucopolysaccharides in certain types of fibrosarcoma would appear to favour this cell type (Gersh & Catchpole, 1949), on the other hand the mast cell is known to store, if not to synthesize, heparin—a non-acetylated sulphuric ester of glucuronic acid—and Asboe-Hansen (1951) has put forward much evidence to suggest that it is also the source of the connective tissue mucopolysaccharides

It is not inappropriate to recall, in this, the centenary of Paul Ehrlich's birth, that his first publication dealt with the mast cell which he so named as it appeared most commonly to be associated with connective tissues whose nutrition had been enhanced Nothing more was learnt about this

ubiquitous cell for sixty years until Jorpes and Holmgren displayed their relationship to heparin and now it seems probable that in addition to their part in the formation of connective tissue mucopolysaccharides, they may well be the source of histamine (Riley 1953). Ehrlich was right to think of them as the well fed cell of the connective tissue, for which an interesting function might one day be found, and it is to be hoped that the time will not be far off when the elastic tissue, which Ehrlich's cousin, Karl Weigert, studied so assiduously, will also reveal its secrets and thus it should be possible to portray a more complete picture of connective tissue—the third estate, as Virchow called it.

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HISTOCHEMISTRY OF CONNECTIVE TISSUE

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Introduction

Histochemistry is used in this review in the broad sense of all the chemical reactions identifying materials in tissues However, most of the histologically precise information is derived from the performance of chemical tests on tissue sections In a very large sense, then, the validity of observations depends upon the chemical specificity of the tests employed While this is such a very important feature, it is nevertheless so large a subject that it would not be possible within this review to evaluate all of the histochemical methods which have been used Excellent considerations of the involved chemical reactions are available in the monographs by Lison (1953), Gomori (1952), Pearse (1953), and Lillie (1948) Many of the methods have been included in a critical review by the author (McManus, 1952)

The term *mesenchyme* as used in this review is synonymous with the term *connective tissues* It includes special cells, special protein fibers and a mucoid ground or intercellular substance of amazing chemical complexity and biological versatility. The cells include fibrocytic and adipose types as well as those of cartilage and bone. Mast cells or tissue basophiles are sufficiently frequent to be considered a normal constituent of the mesenchyme The fibers are collagen, reticulin, and elastic types. The interstitial ground substance shows the specialized feature of calcium deposit as in bone, or high polymerization, as in cartilage, as well as the rarefaction producing the joint spaces and serous cavities

Histochemical methods however precise are not able to identify more than a small fraction of the many important chemicals in the mesenchyme. Sodium, potassium, chloride and other ions, with water making up the *milieu interne*, cannot be demonstrated histochemically The diffusion of

these ions occurs at so rapid a rate that attempts at histochemical localization have been criticized on very reasonable grounds. Similarly, the use of Evan's Blue B (T 1284) to demonstrate the hydration of the mesenchymes has still so many poorly understood features that interpretation of the results must be tentative.

Ground Substance

METHODS

This portion of the review will attempt to summarize objective considerations of the validity of the histochemical methods most in use.

Metachromatic staining is the oldest of the histochemical techniques in use in the study of the mesenchyme There is by no means complete agreement at the present time as to the interpretation of this interesting stain. The term *metachromasia* was introduced by Ehrlich (1877) to describe the phenomenon by which certain tissue structures are stained by dye solution in a color which was different from the color of the dye solutions. Cornil, Jurgens & Heschl had described this property of certain dyes in 1875. The metachromatic property is restricted to basic dyes The most commonly used of these dyes includes thionin, toluidin blue, the azure dyes, and cresyl and methyl violet Lison (1933, 1935) believed that the metachromatic property depends upon a tautomeric form amino base of the dye which is in a labile equlibrium in the dye solution Certain substances, according to Lison chiefly sulphated polysaccharides, have the property of absorbing the metachromatic amino base. Bank & Bungenberg de Jong (1939) suggest that metachromasia does not depend upon a well defined chemical structure but is rather a phenomenon largely governed by electric charge density appearing in the presence of many various materials Michaelis & Granick (1945) believed that the metachromatic color of the dye depends on a polymerization of the dye itself. Michaelis (1947) subsequently altered this viewpoint. Bignardi (1940) and Francini (1940) have shown that glycogen and starch respectively become metachromatic after chromic acid oxidation, while Follis (1951) has shown that the connective tissue of the skin becomes metachromatic after trypsin digestion. Materials such as hyaluronic acid (Blix, 1941, and Wislocki, Bunting & Dempsey, 1947, appear to confirm although Sylvén & Malmgren, 1952, deny this), ribonucleic acid (Flax & Himes, 1952), and hexosemetaphosphate (Wiame, 1947) appear to take the metachromatic stain

Under these conditions it would appear that metachromatic stains give a suggestive type of information which is not yet at the factual stage

Oxidation methods have demonstrated that many of the ground substance materials appear to contain 1,2 glycol groups Chromic acid, as in the Bauer method (1933), and periodic acid (McManus, 1946, Lillie, 1947, and Hotchkiss, 1948), have been used as well as such other oxidants as lead tetra-acetate (Lhotka, 1952 a, Glegg, Clermont & Leblond, 1952, Jordan & McManus, 1952) Lead tetra-acetate appears to have value equivalent to that of periodic acid for the histochemical demonstration of 1,2 glycols provided that the conditions of the special solvents necessary for lead tetra-acetate are appreciated (McManus, 1952, Mowry, 1952) Permismuthate (Lhotka, 1952 b) seems to have similar specific oxidant properties

A great deal of the difficulty in the interpretation of the periodic acid Schiff's results in tissue sections has derived from the variety of methods employed Some of these difficulties have been summarized by McManus & Hoch-Ligeti (1952) Cellobiose was reported to give a negative reaction with the spot test, using the periodic acid Schiff technique of Hotchkiss, by both Hotchkiss (1948) and Jeanloz (1950) McManus and Hoch-Ligeti, however, were able to obtain a positive reaction with cellobiose, using the PAS method as originally described without a reducing rinse

For purposes of clarification of technique employed in various researches it is suggested that the following abbreviations be put into use (1) PAS technique will describe the use of an aqueous solution of periodic acid for oxidation followed by a rinse in tap or distilled water and a period of time in Schiff's reagent This is a method originally described by the author (McManus, 1946, 1948), and to which the term PAS reaction was originally given, (2) APS technique will describe the technique of Lillie (1947) in which periodic acid is formed by the acidification of periodate solutions Exposure of the tissue section to the acidified periodate is followed by Schiff's reagent The factors of stability and definite pH of the acidified periodate deserve investigation, (3) PARS will refer to the reaction of Hotchkiss in which following aqueous periodic acid oxidation a reducing rinse is inserted between the oxidant and the Schiff's reagent It has been adequately shown by McManus (1948), McManus & Hoch-Ligeti (1952) and Lillie (1952 a, 1953) that the reducing rinse will act as a blocking agent for some of the aldehyde groups produced by the periodic acid oxida-

tion. It seems to the author that a very great deal of clarification of a confused literature would be accomplished by such a critical tabulation of the periodic acid methods used in histochemistry.

There seems to be fairly complete agreement that 1,2 glycols are demonstrated in tissue sections by the PAS method, that these are most numerous in materials consisting of carbohydrate or contain a carbohydrate moiety, and that the possibility exists that under certain circumstances (? of disease?) terminal serine, threonine or hydroxylysine groups may be acted upon to produce a periodic acid Schiff reaction. The acetylation and de-acetylation technique depends upon the blockage of 1,2 glycol groups by acetylation and the removal of this blockage by weak alkali. More rapid saponification of the acetylated hydroxyl groups by the weak alkali occurs as compared to the more difficult saponification of the acetylated amine groups This method was introduced into histochemistry by McManus & Cason (1950) but appears to have been suggested also by Gersh (1949)

Iron absorption techniques have been suggested by Hale (1946) for the demonstration of acid mucopolysaccharides in tissue sections Lillie & Mowry (1949) used a colloidal iron solution to study the staining of various tissue structures. As Gomori (1952) and McManus, Lupton & Graham (1951) have pointed out, a blue stain with the Hale method is shown by many structures such as nuclei which could not possibly be acid mucopolysaccharide. *Ritter & Oleson (1950) have combined the* PAS and Hale stains. The complications of interpretation of this technique are considerable. Rinehart & Abul-Haj (1951) have independently produced a stain very much like the Ritter & Oleson which combines the PAS and Hale technique and have further complicated interpretations by using such other staining solutions as Cochineal in the finished preparation. Combination stains of the Hale-PAS type produced some very attractive pictures and are inevitably very much in use in the study of connective tissues.

Pearse (1954) applied the *alkaline tetrazolium reaction* to the study of reducing groups in tissue sections including some of the carbohydrates. The author stresses that this is the introduction of a method whose complete evaluation will require time. Ground substance carbohydrates are reducing sugar containing and should give the reaction as outlined by the author. He found positive reactions among the connective tissue mucins, "possibly including hyaluronic acid and chondroitin sulphate", fibrinoid, etc. Weak reactions were observed in collagen

Alcian Blue, introduced by Steedman (1950) can be made rather selective for acid mucopolysaccharides by lowering the pH of the staining solution (Mowry, unpublished). Selective coloration of many epithelial mucins, connective tissue mucins (umbilical cord, arteriae, and cartilage), as well as capsules of *cryptococcus neoformans* was obtained in formalin-fixed tissue sections stained with 0.1 per cent Alcian Blue in 3 per cent acetic acid (pH 2 6), for 10 to 30 minutes The results are similar to those obtained by the Hale reaction The Alcian Blue method is a single-step procedure and can also be applied in 70 per cent alcohol at pH 1–2 In combination with the PAS reaction, differential coloration of 1,2 glycols and predominantly sulphated or acid mucopolysaccharides may be possible A paper by Lison (1954) explores the usefulness of this new and promising stain, so far little used in histochemistry.

Other staining methods are chiefly of historical interest These include the various mucin stains of Mayer (1896), Bismarck Brown (as re-introduced by Leach, 1947), and Best's stain for glycogen (1906) which represent staining methods whose histochemical bases are worthy of much investigation Ruthenium red, an old method for peptic substances in plants, was used a long time ago by Heidenhain for the study of the ground substance of the ligamentum nuchae. However, in spite of the fact that the similarity of connective tissue ground substance and products to peptic materials the use of Ruthenium red has been unsuccessful in my own hands although the results have suggested some value for it

Enzymatic studies of connective tissue have consisted of (1) the demonstration of enzyme in connective tissue and (2) the action of enzyme on connective tissues The first type of study has been most useful in the study of cartilage, bone and the formation of fibrous tissue. Within the ground substance itself it would appear that the metabolic plasticity of the material is such that enzyme contents will have to be demonstrated in the test tube, as the demonstration of hyaluronidase in the vitreous humour of the eye Concerning the action of enzymes on tissues, reference can be made to the technical books mentioned earlier and to the fact that pure enzyme preparations are extremely difficult to obtain. There is a difficulty of interpretating results rather than a difficulty in obtaining enzymes capable of dissolving one or another material from sections of connective tissue. Benditt & French (1953) have described some of the problems in the use of enzymes of specific histochemical reagents in connective tissues The author's review (McManus, 1952) has mention of some other enzymatic action on connective tissue

RESULTS

It has been reported that ground substance is PAS positive (Gersh & Catchpole 1949). Metachromasia as a property of the ground substance has been described since at least 1911 (Bojerling) although Virchow (1851) himself had indicated the mucoid nature of the ground substance.

In a review Holmgren (1940) traces out the historical development of the information on metachromatic materials in tissues. The first stage in our information is the description of mucus in various tissues by Virchow (1851), Koelliker (1852), Rollet (1858–1860), and Morochewitz (1874–1877). As Holmgren pointed out, after 1900 many authors indicated that the mucoid materials stained in a metachromatic fashion in the anatomical situations described by the earlier workers The relationship between esterification and metachromasia as a matter of discussion probably dates from Bojerling (1911) and Letterer (1932)

Wislocki, Bunting & Dempsey (1947) relate the metachromasia in mammalian tissues especially to the mucopolysaccharides

Various interpretations of the relationship between the metachromatic staining and the PAS reaction, as well as the intensity of each have been made It has been suggested, for example, that the intensity of the metachromasia of the ground substance varies with functional states Lennox, Pearse & Richards (1952) and Loewi (1953) believe that depolymerization of the mucopolysaccharide components of tissue can alter the metachromasia The same suggestion has been made by Persson (1953) who had *in vitro* evidence to suggest that hyaluronic acid produces a significant metachromasia only when highly polymerized. However, a more rational explanation has been produced by Walton & Rickets (1954) who have shown that the metachromasia is not dependent upon polymerization either of the dye molecules or of the acid polysaccharides but upon the solubility characteristics of the dye-substrate complex. The degree of sulphation is thought to be related directly to metachromasia. These findings suggest a re-interpretation of such observations as the reduction of metachromasia in scorbutic granulation tissue (Penny & Balfour, 1949, Persson, 1953).

Mowry (personal communication) has studied metachromasia in dextran preparations attached to slides. Maximally esterified dextran sulphate is markedly metachromatic while dextran itself is not The reverse is true of the PAS reaction. A partly sulphated dextran may be both PAS positive and metachromatic. Gersh & Catchpole (1949) and Gersh (1951) have

described increased PAS positivity in connective tissue in various states such as active inflammation, invasion by tumors, etc. They interpret this as depolymerization of ground substance From the observations of Mowry quoted above it seems highly possible that so simple a thing as desulphation can increase the PAS reaction while decreasing the metachromasia Mowry's studies, as yet unpublished, emphasize the necessity for care in interpretation of metachromasia, the necessity for the use of 70 to 80 % alcohol solutions of dyes and the use throughout the technique of alcoholic solutions, a procedure dating back to Leach's studies with Bismarck Brown, (1947) and the alcoholic PAS method of Mowry (1951). Hale (1953) has examined a number of materials which are weakly reactive to the PAS technique He notes that certain of these can be made to stain more strongly by preliminary treatment with sodium hydroxide solutions He notes that long chain mucopolysaccharides such as hyaluronic acid, mucin, heparin and chondroitin sulfate, show an especially marked increase in color Hale believes it can be shown that the increased PAS reaction is due to making more available more 1,2 glycol groups and their suitable substituted forms rather than affecting sulphate or metaphosphate groups which are responsible for metachromatic properties It is not apparently a saponification

Grishman (1948) reported a study of mucopolysaccharides in normal tissues She used metachromasia with toluidin blue and the Hale stain for iron absorption She found that aorta ground substance was markedly metachromatic and was faintly stained by the Hale method Cartilage ground substance stained by both methods but unlike the aorta ground substance the staining was removed by bull testis hyaluronidase The whole matter of the non-specificity of hyaluronidase has been brought up for discussion by Meyer and others (1941) and by Humphrey (1946) In a subsequent study of tumors Grishman (1952) interpreted the mixed tumor of the parotid as being entirely of epithelial origin and believed that the cartilage-like structures were formed by alteration of epithelial mucin

Altshuler & Angevine (1949) have called attention to the presence of material coloring with the PAS method and metachromatic stains in the Aschoff body of rheumatic fever, a finding of Bunting (1950) and other workers including the present author The group of collagen diseases, referred to in other parts of this volume, have in common the accumulation of a PAS positive, metachromatic material in the characteristic lesion The skin accumulations of myxedema are largely PAS

demonstration of reducing groups by tetrazolium which are not blocked by benzoylation The same author reports variable metachromasia of variable degree while Follis (1951) reported marked metachromasia after trypsin digestion. Pearse reports a positive PAS reaction after diastase which brings up the continuing argument about the PAS positiveness of collagen Leblond (1950) did not stain with the PAS reaction but he used the Hotchkiss method including the reducing rinse. Wislocki (1952) mentions the intense reaction of collagen with the periodic acid Schiff reagent in many connective tissues. He used "slight modifications of the McManus and Hotchkiss methods" Lillie (1953) re-investigated the problem and believed that the reactivity of collagen to the PAS procedure was lessened by potassium dichromate in fixation Bangle & Alford (1954) re-investigated the problem in sections of human skin and in hide collagen. They believed that the slight coloring encountered in collagen could be explained by the oxidation of non-glucosamine polysaccharide complex conjugated with collagen. They did not believe that lipid or glycogen or the three amino acids susceptible of periodate cleavage were responsible for the PAS positivity of collagen. In my own experience the PAS reaction has been very slightly positive in human collagen under normal conditions.

Reticulin presents a very much different picture. Positive reactions with reticulin have been produced by the PAS technique by many authors, initially by Lillie (1947) In paraffin sections and in celloidin sections after the usual fixatives the demonstration of reticulin in the spleen and very frequently in the lymph nodes is so good that the PAS method has been recommended as a staining technique for reticulin (Lhotka & Davenport, 1950) The situation is confused by Gersh's observation that reticulin fibrils are not demonstrable in his freeze-dry material (Gersh, 1950) In this study he was using the Hotchkiss method and it may be that the reducing rinse quenched the PAS positivity which only in the spleen is very marked The presence of a PAS positive material in reticulin seems to fit in with the possible mode of formation of reticulin but there are no features apparent in the electron microscope to confirm this impression This may, however, be due to conditions of tissue handling preparatory to electron microscopy.

Elastic Tissue

Elastic tissue has relatively been an unexplored area. It gives a pseudo-plasmal reaction especially in rodents while coloring deeply with the al-

dehyde fuchsin stain of Gomori (1950). Like collagen many workers have reported a positive coloring of faint degree with the PAS reaction. Scott & Clayton (1953) point out that mast cells, hyaline cartilage and elastic fibers which contain and are associated with highly sulfated mucopolysaccharides react strongly with aldehyde fuchsin This observation is given emphasis by the studies of Hall, Reed & Tunbridge (1952) These workers find that polysaccharide and sulphuric acid are intimately associated with protein in elastic tissue Particular emphasis is given to the fact that polysaccharide is liberated with protein whenever elastic tissue is degraded, suggesting a close association of these materials The elastase of Balo & Banga (1949) is thought by these authors to be a mucinase or mucase The authors recall that Schultz (1922) had actually suggested that the staining capacity of elastic tissue was due to imbibed mucopolysaccharide

In elastic tissue staining some progress is being made in the demonstration of a histochemical basis for our histological staining methods Orcein introduced by Unna (1890) for the staining of elastic tissue has been separated into four active fractions by Engle & Dempsey (1954) The further studies by Weiss (1954) have shown fairly certainly that staining of elastic tissue by orcein in acid alcohol solution is due to hydrogen bonds Elastin has a low positive charge in acid alcohol solutions which explains the selective reaction with the positively charged orcein fractions For a review of the chemistry of orcein staining, the papers by Engle & Dempsey, by Dempsey et al (1952), and by Weiss (1954) are valuable

Lansing et al (1950, 1951, 1952 a, b) have studied the content of aortae particularly in respect to calcium and amino acid composition A progressive increase in the quantity of inorganic materials with advancing age is a particularly marked feature

Lansing (1951) has a most constructive review on the chemical morphology of elastic tissue He indicates that there are chemical differences between old and young elastins, notably a decrease in the glycine, valine and alanine in the old compared to the young elastin (Lansing et al, 1951) On the other hand, glutamic acid, glycine and arginine show considerable increases The explanation of this is not at all clear The characteristic amino acids of elastic tissue, leucine, isoleucine and valine do not appear to show any striking change with age There is a discussion of the staining and chemical features of arterial elastic tissue which is worth study by anybody interested in the problem Lansing says that the

elastic tissue in human skin is PAS positive. I have not studied this matter particularly but in some unpublished observations it is noteworthy that the elastic tissue of the adrenal medulla in the human is PAS positive also.

Mast Cells

The mast cells of connective tissue are characterized by the possession of cytoplasmic granules stained metachromatically and which in the human usually gives a positive PAS reaction. (Jorpes, Werner & Åberg, 1948). On the basis of these data the workers concluded that the mast cell granules may be heparin precursors. Asboe-Hansen (1950 a, b and c, 1951) found a correlation between the number of mast cells and the quantity of hyaluronic acid in tissues On the other hand, there did not appear to be any definite relation between the sulphomucopolysaccharide content and the number of mast cells It was suggested that the mast cells secrete hyaluronic acid Asboe-Hansen (1953) studied the uptake of radioactive sulphur by the mast cells in experimental skin tumors in mice. Autoradiography of connective tissue in these experimental skin tumors showed that the majority of the mast cells take up the S^{35}. Lillie had noted earlier that in the rat more mast cells were shown with metachromatic staining methods than can be demonstrated with the PAS method. Bensley (1950) studied the histological changes in the ground substance following the injection of testicular extracts locally Immediately mast cells were disrupted and appeared to be reduced in number by 24 hours. By the fourth day there was an increase in number and granulation of the mast cells and at the end of a week there seemed to be an increase in mast cells. Bensley suggests that the mast cell may be part of an enzyme-substrate complex.

Cramer & Simpson (1944) have described an accumulation of mast cells at the margins of tumor invasion. Aykroid & Zuckerman (1938) believed the mucopolysaccharides in the sex skin swelling of the monkey could have originated in the mast cells Sylvén (1941) extends this concept to many varieties of connective tissue The presence of accumulations of mast cells in many skin diseases and their relationship to urticaria pigmentosa where there is increased capillary fragility has many physiological implications which are discussed elsewhere (p. 294).

Riley (1953 a) has taken a new line in the study of the mast cells of the rat. He describes a granulated orthochromatic variety which is most numerous in relation to the blood vessels of the connective tissue and which

he calls type one. The type two mast cell of Riley is a group of mast cells which contain metachromatic granules. These are larger and irregular in shape. Riley believes that the type one mast cell represents the early stage of development while the type two is the more mature variety. The function of the mast cell suggested by these data according to Riley would suggest a physiological role in relation to connective tissue rather than in relation to the blood stream

Pearse (1953) believes that only a proportion of the mast cell granules give a positive PAS, using Hotchkiss' reducing rinse These and the other observations quoted which would suggest variable PAS positivity would be in accordance with a metabolically active cycle within the mast cell granules The mast cell granules color for lipids like the other granules in leucocytes (Sheehan, 1939, McManus, 1946) although Lillie (1953) has seriously questioned the Sudan black staining of the white cell granules as specific for lipids However, Pearse (1953) has shown that secondary fluorescence in ultraviolet light can be induced by the benzpyrene method of Berg (1951).

The comparative staining reactions of the mast cell granules in various animals has not yet been made in an organized fashion Compton (1952) found that in the hamster mast cell granules are more infrequently stained with PAS than they are stained by metachromatic methods. Sudan black B does not stain the majority of the mast cell granules unless they are near fat cells Connective tissue spreads examined with the phase microscope show no evidence of a secretory cycle involving degeneration and death of the mast cell granules The relationship to heparin which has been emphasized by Jorpes (literature Jorpes, 1946) has suggested to Jorpes that the mast cells of tissue should be called heparinocytes. Even at this time the evidence is suggested rather than proven Reference is made to the previous article of Asboe-Hansen and a recent survey of the literature appears in Compton (1952)

Drennan (1951) describes the mast cells in urticaria pigmentosa He describes the variety of appearances which mast cells may have. Whereas Drennan generally found more mast cells in other cases by the toluidin blue staining method rather than with the PAS technique, in urticaria pigmentosa, equal numbers of mast cells were demonstrated by the two staining methods Vacuolation and disruption of cytoplasm are described by Drennan in the active lesions. This is in line with the observation of Riley (1953 b) that vacuoles appear in mast cells with stimulation by histamine

liberators and that this vacuolation can be prevented by antihistamine premedication.

Sundberg et al (1954) describes apparently for the first time, numerous mast cells in Wharton's jelly of the human umbilical cord. Besides the mast cells, both fibroblasts and connective tissue cells are said to contain metachromatic granules Katzberg (1954) describes the distribution of mast cells in the lymphoid organs of the rat. The spleen was almost devoid of mast cells while both thymus and lymph nodes contained a few. In the thymus the mast cells were in the capsule and connective tissue while in the lymph node they were found in the node substance Padawer (1954) repeats his earlier descriptions of mast cells of the rat peritoneal fluid, adrenalectomy reducing mast cell size while hypophysectomy increasing it. Hess (1954) describes ground substance in the developing central nervous system and proposes a role for it in development and in cerebral edema. Constantinides & Rutherdale (1954) report the effect of various materials and maneuvers on the rat mast cell count.

Riley (1953 c) first suggested the mast cell as a site of histamine production Fawcett (1954) reports experimental observations suggesting histamine in mast cells Benditt et al. (1954) report that an edema producing dose of ovomucoid damages and disrupts mast cells at the same time that histamine is released locally. This is taken as further evidence for Riley's suggestion of association of histamine with mast cells

Mast cells in relation to tumors and tumors composed of mast cells—mastocytomas—are reviewed by Cambell (1952) who reports a mastocytoma followed methylcholanthrene painting of the skin of Swiss albino mouse Sabrazès & Lafon (1908) apparently first described this type of tumor in a horse Bloom (1942) reviews the literature and describes mast cell tumors in the dog The tumors of the mastocytoma type assume importance in view of the ideas of Riley regarding histamine and mast cells Riley (1953 c) was able to demonstrate in four mast cell tumors, two from children and two from dogs, extremely high values of histamine. According to Riley, the mast cells are rich in histamine as well as containing heparin. Earlier, Riley & Drennan (1949) seem to have shown alkaline phosphatase activity in the granules of the mast cells.

Twort & Twort (1930) described diffuse mast cell increases in the skin of mice painted with carcinogenic agents as opposed to the nodular "mastocytomas" following tar painting observed by Schreus (1924) and Fabris (1927). Cramer & Simpson (1944) considered the mast cells

as part of a defensive reaction or mechanism Cambell (1952 a, b) agrees with this concept

In other interesting observations Cambell (1952 a, b) noted mast cell diapedesis in the stomach of the albino rat corresponding to the leucocyte gastric diapedesis described by Tomenins (1947)

Fibrocytes

The fibrocyte is the most numerous cell in the body ground substance It varies considerably in size and shape and may contain inclusions of fat or protein and commonly of carbohydrate Gersh & Catchpole (1949) have described the presence of the PAS positive granules in the fibroblastic cytoplasm relating this to alterations in the PAS positivity of the ground substance Marked cellular hyperactivity shows no PAS positive granules in the fibroblasts The carbohydrates and lipids of the fibroblasts occur in the classical position of the Golgi element of the fibroblasts (unpublished observations) This suggests a relationship to metabolic activity Cytoplasmic basophilia in the fibroblast, associated with an increase in material removable by ribonuclease occurs in conditions of increased cellular activity, as in repair, tumor formation, etc. There appears to be some relationship between the granulation and frequency of the mast cells and the basophilia and activity of the fibrocyte The presence of fibrils within the cytoplasm of the fibroblasts in tissue culture as shown by Bang & Gey (1948) and by Porter (1951) suggests that the fibroblast is concerned with the fibrillar element of connective tissue rather than with ground substance

Miscellaneous Observations

De Brux (1951) reviews the histochemical constitution of the ground substance and relates this to the "collagen diseases" He reviews the evidence that a secretion of fibroblasts makes up one of the most important factors in the formation of the ground substance The influence of various enzymes, of the hormones, on the breakdown of the ground substance is entered into in considerable theoretical detail De Brux & du Boistesselin (1953) enlarge upon the alterations recognizable by histological and histochemical methods which occur in the connective tissues under the action of various hormones Kellgren (1952) has summarized the study of his group and others in the rheumatic diseases Corresponding to the fibrinoid

change of collagen in rheumatoid arthritis, they have found an actual decrease in the ground substance of cartilage exposing collagen fibers in the fraying process of the osteoarthritic joint. Accessory evidence for the carbohydrate nature of the fibrinoid materials is presented in this review.

There has been a very considerable amount of interest derived from the coloring of amyloid infiltrations by the PAS method (McManus, 1948). The variety of other connective tissue hyalines which color with the PAS method includes that of arteriosclerosis, the hyaline filling the space of Bowman's capsule in ischemic obsolescence of glomeruli and in the hyaline of Zenker's degeneration of muscle (McManus & Cason, 1950). In regard to the hyaline in Bowman's capsule it has been reported that a concentration of 5-nucleotidase is present in the abnormal material (McManus & Lupton, 1953). Ehrich (1952) discusses some of the relationships between pathological hyalines

Hudack & Blunt (1950) describe one of the early investigations of wound healing using a variety of histochemical techniques. They refer to an earlier observation by Hudack et al (1949) on what was thought to be an acid mucopolysaccharide present in the granulation tissue of healing fractures. Higbee (unpublished data) is said to have correlated its presence with fibroplasia, the material disappearing nearly completely in fully mature fibrous tissue or bone. Hudack & Blunt quote some unpublished work of their own in which there was increased phosphatase activity, lysozyme, sulphate radicals and implied phosphate enrichment. Layton (1950) reports the *in vitro* fixation of sulphate radical by granulation tissue. Stoughton & Wells (1950) have reported the histochemical study of various skin diseases. McManus & Lupton in unpublished studies have made a histochemical survey of the wound healing in rabbits and rats. While the details are so numerous that a complete summary would be out of place certain broad principles were apparent. It appeared that the healing in cartilage had many similarities to the healing in the loose connective tissue with the major difference that the ground substance polysaccharides were much more viscous in the cartilage and could be readily followed. If there was no great displacement in the wound between the fragments of cartilage, granulation tissue did not form and inflammation was at a minimum The ground substance of the cartilage appeared to exude to fill up the gap and this was quickly invaded by perichondrial cells and cells from the adjacent cartilage. These more active cells did not contain the readily fixed and preserved carbohydrate, probably glycogen, of the resting mature cartilage

cells The ground substance was less PAS positive in the gap between the ends of the cartilage, it took more of the Hale stain and was more metachromatic. As this healing cartilage matured, it became more PAS positive, the cells became more filled with glycogen and the staining reactions returned in time to those of normal cartilage tissue. Displaced portions of cartilage were re-absorbed with the early disappearance of the ground substance while the elastic tissue persisted This process of healing in cartilage is in all probability a prototype of the healing by first intention as it occurs in the human skin wound.

Brewer (1951) reports precise autopsy histochemical study of a case of myxedema. Mucoid infiltrations were studied in the skin, the tongue and the myocardium It was concluded that the infiltration of the tongue was probably a mixture of protein containing hyaluronic acid and chondroitin sulphuric acid whereas the infiltration of the myocardium consisted of "a histochemically distinct mucoprotein" The literature on mucoid degeneration of the myocardium (Spencer, 1950) is reviewed by Brewer The mucoid degeneration of Spencer is the same as the basophilic degeneration of the myocardium of a number of authors

Campani & Reggianini (1950) review the Italian literature on metachromasia and point out that in a healing wound metachromatic ground substance can be removed by hyaluronidase up until the fourth day. After the fourth day the metachromasia is not removable by hyaluronidase, these observations suggesting strongly some essential change in the metachromatic material They suggest that the metachromasia which is earlier removed by hyaluronidase is probably due to hyaluronic acid which may become more polymerized and resistant to hyaluronidase in the latest stages of healing Balasubrahmanyan (1953) describes a PAS positive interfibrillary substance in early carbon tetrachloride cirrhosis This PAS positivity can be partly removed by hyaluronidase. As the fibrous tissue gets older the PAS positive material decreases in amount

Cater (1951) has studied by histochemical and biochemical methods some effects produced by the Rous sarcoma upon the endocrine organs of cocks and hens A variety of methods were used including ascorbic acid, lipids, alkaline phosphatase, acid phosphatase and the PAS method A variety of changes are described which should be sought in the original publication

Robertson, Dunihue & Novikoff (1950) were not able to be certain that increased alkaline phosphatase was ascociated with fibrous tissue

formation This article reviews some earlier contrary opinions. About the only other enzyme considered important in connective tissues from a histochemical viewpoint is the β-glucuronidase of Fishman (1940). Burton & Pearse (1952) present a critical study of the histochemical methods for its demonstration. Campbell (1949) described an increase of β-glucuronidase activity demonstrated histochemically at the sites of active tumor growth

Difficulties in interpretation of histochemical studies are apparent in attempts at deciding the origin of cartilagelike materials in the so-called mixed tumors In those of skin origin, Lennox, Pearse & Richards (1952) believe that epithelial mucin may give rise to cartilage. The immediately following article by Yates & Paget (1952) expresses the belief that the cartilage in the salivary gland mixed tumor develops from the connective tissue stroma.

In relation to the descriptions of Hess (1954) above, the disease metachromatic leuco-encephalopathy of Einarson & Neel (1938) comes to mind. Leslie (1952) has studied such a case by histochemical staining methods It is believed that the granular deposit is a glycolipid.

In summary, this review has examined the methods most used in the study of the histochemistry of connective tissue The data produced in a large number of such studies have been described and a broad outline of the histochemical structure of the mesenchyme has been presented. The important position of the mast cell in modern considerations of connective tissue seems especially striking This may be more related to its ease of demonstration than to anything else Large deficits in our knowledge of the histochemistry of the mesenchyme include enzymatic activities, the lipids and comparative data obtained by carefully controlled studies

This review has the limitation of available page space with the additional restriction, self-imposed by the author, that recent, crucial, original or review articles have been the main sources of material. The omission of undoubtedly important articles has been forced by one or another of these restrictions

Summary

The connective tissue from the viewpoint of histochemistry is made up of protein fibers of several specific types embedded in a mucous ground substance. The protein fibers are of three main types: 1) Elastica, 2) Collagen and, 3) Reticulin The first two of these fibers possess no specific

histochemical features; methods for their demonstration are histological and empirical, although possessing a high degree of specificity. The third variety of fibers, reticulin, contains carbohydrate and can be demonstrated by various oxidative methods

The ground substance may be demonstrated by oxidation methods and by metachromatic staining The present opinion suggests rather strongly that neither of these methods of demonstration depends upon the hyaluronic acid of the ground substance but rather upon other materials including chondroitin sulphuric acid compounds.

The fibroblasts and mast cells are the most important cellular constituents of the connective tissues It appears from histochemical data that the fibroblast is related especially to the fibrous elements of the connective tissues while the mast cells reflect and/or cause changes in the ground substance These changes include increased or decreased metachromatic staining, frequently accompanied by changes in intensity of staining by oxidative methods The interpretation of these data is difficult but the changes are thought to represent the degree of polymerization, changes in viscosity, or even such a simple factor as degree of sulphation The mast cells themselves are associated with sulphate metabolism, heparin and/or hyaluronic acid and histamine release They represent the present points of most intensive study

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THE CHEMISTRY OF THE GROUND SUBSTANCES OF CONNECTIVE TISSUE

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Introduction

INFORMATION ON THE COMPOSITION of the ground substances of connective tissue is based on histological data, on chemical isolation and, to a minor extent, on physiological studies All these methods have severe limitations. The histological methods in this field have very limited specificities, even when used in conjunction with enzymatic digestion. Chemical isolation, on the other hand, requires large samples and fails to give information of the topology of the substances isolated. In fact, the proposition of the origin of the isolated substances from the interfibrillar spaces, though very probable, is only based on assumption The physiological methods have given very limited information on the structure and chemical nature of the ground substances.

It has become evident in the last few years that the ground substances are highly complex and differ in composition in various organs. In fact, only in synovial fluid and vitreous humor has a single component been found—hyaluronic acid From all other tissues, two or three different mucopolysaccharides have been isolated. It is, of course, arbitrary to consider synovial fluid or vitreous humor as analogous to mesodermal ground substances as has been done by various authors in the case of synovial fluid In the case of the vitreous humor, it is even uncertain whether its origin is mesodermal.

Analysis of the Structure of the Ground Substances

HISTOLOGY

It is not possible to deal with the chemistry of the ground substances without making some statement as to their histology. The work of Bensley (1950), Day (1952), and Persson (1953) has provided a picture of loose connective tissue as forming a fabric composed of lamellae or fibres, the interstices of which contain a plastic fluid Dense connective tissue such as tendon and large blood vessels must have a structure unlike that of loose connective tissue. It contains a lower mucopolysaccharide concentration in which the chondroitin sulfates predominate The relation of the latter to the fibrous elements is unknown, but it can be assumed that they are structurally and functionally intimately connected

The structure of reticulum apparently is quite different from that of the collagenous components of connective tissue (Benedetti, 1953) The ground substances of reticulum may also be different. By paper chromatography, galactose and mannose, fucose and ribose have been demonstrated in hydrolysates of the residues of water and acetone-extracted tissues rich in reticulum fibres (Glegg et al, 1953) Galactose, mannose and fucose have also been demonstrated in cornea (Werner & Odin, 1949) It seems imperative that these reports should be confirmed by chemical identification and especially that quantitative data and isolation of defined fractions should be forthcoming

CHEMISTRY

(a) *General* For the isolation of components of the ground substances of connective tissues, extraction with dilute alkali or aqueous salt solutions of fresh or dried tissues has been employed Digestion with proteolytic enzymes has been substituted by a number of authors for the alkaline extractives Depending on the tissue and the method of extraction used, the extracts contain protein and other nitrogenous components, neutral sugars, mucopolysaccharides and mucopolysaccharide-protein complexes. The proteins partly consist of collagen When a salt solution or mild alkali is used as an extractive, the sulfated mucopolysaccharides are obtained as protein complexes The nature of the bonds between protein and mucopolysaccharide is still uncertain In the case of hyaluronic acid, this author has considered these bonds to be of polar type (Meyer, 1947). Ogston and Stanier (1953) on the basis of viscometric studies have postulated that,

in ox synovial fluid, hyaluronic acid is present as a protein complex composed of about 30 per cent protein and 70 per cent hyaluronate. The nature of the bond has not been discussed by these authors. They attribute the physical properties, however, especially viscosity, to the intactness of this linkage. Blumberg & Oster (unpublished) studied light scattering, sedimentation, and streaming birefrigence of hyaluronate prepared by this author from umbilical cord On the basis of these experiments, these authors arrived at a molecular weight, shape and properties of the compound not significantly different from those reached by the Oxford authors The preparation used, however, contained less than 0.3 per cent protein based on the ratio of nitrogen to uronic acid

Chondroitin sulfate B and C appear to be bound to protein in the connective tissues much more firmly than is hyaluronate. Thus from extracts with aqueous 2 per cent phenol or with 0 02 N $Ca(OH)_2$ fractions *were obtained consisting of approximately* 25 *per cent mucopolysaccharide* and 75 per cent protein which migrated electrophoretically at pH 8 6 as complexes On treatment with strong alkali, the complexes dissociate into their components

Chondroitin sulfate exists in cartilage at least in part in polar linkage and migrates electrophoretically free of proteins (Blix, 1940) From acetone and ether-extracted cartilage powder, the mucopolysaccharide can be extracted almost quantitatively by neutral or slightly alkaline $CaCl_2$ solution (Meyer & Smyth, 1937) It apparently has been assumed that chondroitin sulfate is bound to collagen in cartilage, an assumption which may not be warranted

(b) *The Mucopolysaccharides.* While the protein components of the ground substance have scarcely been studied, the mucopolysaccharides of these tissues have received a great deal of attention The following acid mucopolysaccharides have been isolated:

1. hyaluronic acid
2. chondroitin sulfate A
3. chondroitin sulfate B
4. chondroitin sulfate C
5. chondroitin
6. keratosulfate

The following table illustrates the distribution of these polysaccharides based on their isolation

This table contains all available data. It is probable that these may have to be modified and extended as some of the tissues are more carefully

TABLE 1

	Tissue	Hyaluronic acid	CHS A	CHS B	CHS C	Chondroitin	Keratosulfate
Group I	Vitreous humor	+	---	---	---	---	---
	Synovial fluid	+	---	---	---	---	---
	Mesothelioma	+	---	---	---	---	---
Group II	Hyaline cartilage	---	+		+	?	---
Group III	Heart valves	---	---	+	+	?	
	Tendon	±	---	+	+	?	
	Aorta	---	---	+	+	?	
Group IV	Skin	+	---	+	---	?	---
	Umbilical cord	+	---	---	+	?	
Group V	Cornea	---	+	---	---	+	+

examined and as new tissues are being studied In the following, the individual mucopolysaccharides will be discussed

1 Hyaluronic acid (The history and properties of hyaluronic acid have been reviewed (Meyer, 1947) and this discussion will be limited to information not covered previously) Hyaluronic acid can be defined as a polymer of a disaccharide N-acetylhyalobiuronic acid polymerized, probably as unbranched chains, via the glucosaminidic bonds (Weissmann & Meyer, in press). It is apparent from viscometric data that the polysaccharide occurs in nature in various degrees of polymerization The only material studied extensively is that isolated from umbilical cord which has, by the light scattering method, a molecular weight of approximately 8 million (Blumberg & Oster, unpublished). The shape of the particle in solution appears to be formed by coiling of highly hydrated chain molecules of about 10 Å thickness, forming hydrated spheres of a diameter of 2100 Å Hyaluronate of high purity prepared from a group A hemolytic streptococcus (Blumberg, Halbert & Meyer, unpublished) appears to have properties very similar to that of umbilical cord and may have a still higher molecular weight The chemical structure of hyaluronic acid has been investigated by three groups by the use of periodate oxidation (Meyer, K H et al, 1951; Jeanloz & Forchielli, 1951 b, Blix, 1951). The conclusions of these three groups contradict each other. It is quite obvious from the work of Jeanloz and Forchielli (1951 a) that periodate oxidation of glucosamine derivatives under controlled conditions does not yield the expected stoichiometric quantities. In this laboratory, the structure of the

repeating disaccharide unit of hyaluronic acid was shown to be 3-β-D-glucopyruronosyl-2-acetamino-2-desoxy-D-glucose (Weissmann & Meyer, 1952; Weissmann et al., 1953). The structure was proven by demonstrating the identity of an acetylated degradation product of the latter with the corresponding acetyl derivative from laminaribiose, a synthetic disaccharide of proven structure. In this work, hyalobiuronic acid was esterified and reduced to the corresponding D-glucopyranosyl compound. The latter was desaminated and decarboxylated to the 2-β-D-glucopyranosyl-1-D-arabinose, *isolated as the heptaacetyl compound. A heptaacetate of the* same melting point and optical rotation was obtained from laminaribiose by Zemplen degradation.

Evidence for N-acetylhyalobiuronic acid as the main if not only building block of hyaluronic acid is based on the following findings The crystalline disaccharide, hyalobiuronic acid, was isolated in a yield of 61 per cent by acid hydrolysis of digests of hyaluronate by testicular hyaluronidase. By direct acid hydrolysis of hyaluronate, the disaccharide was isolated in somewhat lower yields (Weissmann & Meyer, in press). On acetylation with ketene, the amorphous N-acetyl derivative of hyalobiuronic acid was obtained which, in its physical properties, was indistinguishable from the disaccharide fraction obtained from hyaluronate by hydrolysis with purified or crude testicular hyaluronidase. N-acetylhyalobiuronic acid, prepared both enzymatically and synthetically, yielded identical, well characterized, heptaacetyl methyl esters (Weissmann et al., in press). The evidence for an unbranched chain in hyaluronic acid was based on the results of the digestion of hyaluronate by hyaluronidases (Meyer & Rapport, 1952). There is as yet no conclusive evidence for the nature of the hexosaminidic bond by which the disaccharide is polymerized into hyaluronic acid. It is, however, probable that this bond has a β-1-3 configuration. This conclusion is based in part on the absence of periodate consumption of hyaluronate, in which the three investigators mentioned previously agree. The assumption that this negative evidence demonstrates the absence of vicinal hydroxyl groups in conjunction with the demonstrated pyranose structure and 1-3 linkage of the uronidic moiety, leaves for the glucosaminidic moiety only a 1-3 linkage in the pyranose configuration On the basis of the strong negative rotation of the polymer ($[\alpha]_D$ —70°), this linkage can also be assumed to be in β-configuration. These data are summarized in the following structural formula for hyaluronic acid

Hyaluronidases. The enzymes hydrolysing hyaluronate have been used

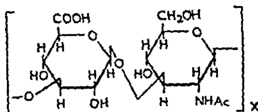


Fig 1

and are still being used in the study of hyaluronic acid and of connective tissue in general For this reason, it may be appropriate to summarize pertinent data about the enzymes and their action on hyaluronate and other mucopolysaccharides It is, of course, possible to give only a general survey of this field in the framework of this presentation Of the two glycosidic bonds in hyaluronic acid, only the glucosaminidic bond is attacked by hyaluronidases, that is, all hyaluronidases are hexosaminidases. All hyaluronidases thus far studied appear to hydrolyse the polysaccharide either randomly or from the center No enzyme has yet been found attacking hyaluronate from the end analogous to β-amylase. All hyaluronidases thus far studied attack the native high polymers as well as the polymers of lower molecular weight, although enzymes of various origin may differ in their affinity to the substrate. Hyaluronidase activity, whether measured by its biological effects such as spreading activity or by its physical effects like loss of mucin clot formation and loss in viscosity, or by its chemical effects like increase in reducing sugar value, appears to be due to only one reaction, the opening of glucosaminidic bonds (Meyer & Rapport, 1952)

Two main groups of hyaluronidases can be distinguished, those of animal origin (A) and those of microbial origin (B). The best studied members of group A are testicular hyaluronidase and, though studied much less, snake venom hyaluronidase (Linker & Meyer, unpublished). Group B hyaluronidases which have been studied are derived from pneumococci, streptococci, staphylococci and clostridium Welchii (Linker & Meyer, unpublished).

The following table lists some of the properties of the two groups of enzymes

The most striking differences listed in Table 2 in the two groups of enzymes are 1) the extensive transglycosidase activity of hyaluronidase A,

TABLE 2

	Animal hyaluronidases or hyaluronidases A	Microbial hyaluronidases or hyaluronidases B
Activity	endo-β-hexosaminidase, extensive transglycosidase	endo-β-hexosaminidase, no transglycosidase
Stability	thermostable	thermolabile
Substrate affinity	increases with molecular weight	nearly independent of molecular weight
Endproducts	disaccharide and oligosaccharide with tetrasaccharide predominating	disaccharide
Disaccharide endproducts	β-glucopyranurosido-1-3 N-acetyl D-glucosamine	modified from normal disaccharide by H-OH loss, presumably β-D-gluco-4-5 ene-pyruronido 1-3 N-acetyl D-glucosamine

and 2) the endproducts of the action of the two groups of enzymes Another difference not listed above is the hydrolysis of chondroitin sulfate A and C by testicular hyaluronidase and the resistance of these compounds to the bacterial enzymes. This resistance, however, disappears when the sulfate groups are removed by acid hydrolysis (with partial concomitant hydrolysis of glycosidic bonds) (Davidson & Meyer, unpublished) or more completely with the naturally occurring chondroitin (see below). The prolonged hydrolysis of hyaluronate by purified testicular hyaluronidase was shown to lead to a mixture of oligosaccharides, which by paper and ion exchange chromatography was resolved into a consecutive homologous series ranging from di- to octadekasaccharide. Exhaustive hydrolysis led to a mixture containing 70 per cent tetrasaccharide, the rest composed of disaccharide and higher homologues (Weissmann et al., in press) Presumably the enzyme which hydrolyses is also responsible for the transglycosidation. In this reaction, disaccharide units or multiples of disaccharide are transferred to higher units (Weissmann & Meyer, 1953). Di- and tetrasaccharides apparently do not act as acceptors, but the tetrasaccharide may be hydrolysed, although very slowly. The transglycosidase reaction occurs concomitant with the hydrolysis. The rate of both reactions appears to increase with increasing molecular weights of the oligosaccharide fractions The transglycosidation probably leads to the production of the consecutive homologous series of multiples of disaccharides obtained in non-exhaustive hydrolysis.

The endproduct of the action of microbial hyaluronidases contains equimolar quantities of hexosamine (determined colorimetrically) and of uronic acid measured both colorimetrically and by CO_2 determinations The bacterial disaccharide has now been shown to contain a hitherto undescribed type of 4–5 unsaturated uronic acid, apparently formed by the removal of H–OH during the hydrolysis of the glucosaminidic bonds. Thus, the tetrasaccharide (the main product of testicular hyaluronidase action on hyaluronate) yields equal quantities of normal and bacterial disaccharide (Linker et al., 1954) on incubation with bacterial hyaluronidase while N-acetylhyalobiuronic acid remains unchanged on incubation with the enzyme

Apparently a similar conversion of the normal glucuronide to the unsaturated uronide occurs when the substrate is chondroitin or partly desulfated chondroitin sulfate (Linker & Meyer, unpublished).

When the hydrolysis of hyaluronate is interrupted before completion, the oligosaccharide fractions produced all appear to contain the glucosenuronic acid end group The oligosaccharides have paper mobilities different from the normal oligosaccharides and they are resistant to β-glucuronidase, while this enzyme hydrolyses the terminal glucuronidic groups of the normal oligosaccharides (Linker & Meyer, 1952).

The biosynthesis of hyaluronate from labelled glucose has been studied in group A hemolytic streptococci (Topper & Lipton, 1953, Roseman et al, 1953) This organism utilizes glucose directly for the synthesis of both the glucuronidic and glucosaminidic moiety. Recently the same technique has been used for the study of skin hyaluronate synthesis in the living rabbit. The mechanism of the synthesis is as yet unknown.

2 Chondroitin Sulfate A Chondroitin sulfate A is the main mucopolysaccharide component of hyaline cartilage. In hyaline cartilage a minor component occurs which has the solubility and rotation of chondroitin sulfate C Chondroitin sulfate A also has been prepared from a cystic human chondrosarcoma (Meyer, unpublished). The only other known source of a compound analogous in its properties to chondroitin sulfate A is the cornea (Meyer et al, 1953) Chondroitin sulfate A is a polymer with little or no branching composed of equimolar quantities of N-acetyl chondrosamine (D-2-acetamido-2-desoxy-galactose, Foster & Stacey, 1952), glucuronic acid and sulfate As with hyaluronate on acid hydrolysis, a desulfated and deacetylated disaccharide has been obtained in excellent yield, the so-called chondrosine which on esterification yields

the crystalline methylester (Levene, 1941) Recently in this laboratory, crystalline chondrosine has been obtained. The structure assigned to this disaccharide is analogous to hyalobiuronic acid. It is β-glucuronido-chondrosamine (Davidson & Meyer, unpublished) and not chondrosaminido-glucuronic acid (Wolfrom et al., 1952). The sulfate groups in chondroitin sulfate A presumably is in the hexosamine moiety, although no strict proof of this is available. Chondroitin sulfate A is usually prepared from either tracheal cartilage or from nasal septum. It is advisable, especially with tracheal cartilage, to remove loose connective tissue, muscle and other contaminating tissues by a short incubation with acid pepsin, followed by mechanical removal of the extraneous tissue. (For literature on the preparation, see Mathews & Dorfman, 1953). Recently the preparation of the Ca salt in crystalline form has been reported (Einbinder & Schubert, 1951). The best preparations of chondroitin sulfate A (including that of the crystalline Ca salt) usually show a molar sulfate ratio slightly below that of hexosamine and uronic acid. The optical rotation of the neutral salts is $[\alpha]_D = -30°$ to $-32°$, that of the acid salts $-17°$ to $-19°$. The molecular weight of chondroitin sulfate A was estimated from streaming birefrigence data as 260,000 (Blix & Snellman, 1945). Alkali-treated chondroitin sulfate lost its streaming birefrigence and its molecular weight dropped to about 50,000. Considerably lower molecular weights as determined by osmotic pressure were reported for chondroitin sulfate A prepared by a variety of methods (Mathews & Dorfman, 1953; Mathews, 1953). The highest value, 43,000, was obtained from $CaCl_2$ extracted samples The samples showed weak flow birefrigence only in water and none in buffer solutions. Alkali-treated or KCl-extracted material showed molecular weights as low as 15,000. The discrepancy between the data of Blix and of Mathews is unexplained. The degree of purity of the preparations of the two laboratories is about the same as judged by the reported analyses. One sample prepared in this laboratory from tracheal cartilage also showed little flow birefrigence (Blumberg & Oster, unpublished). On ultracentrifugation, it appeared polydisperse

Chondroitin sulfate A has been reported to consume 1 molecule of periodate per disaccharide unit (Wolfrom et al., 1952; Meyer et al., 1948). Blix (1951), however, reported a low periodate consumption. It should be noted that the first two authors had undoubtedly degraded chondroitin sulfate Very little is known about the biosynthesis and breakdown of any of the chondroitin sulfates. The fixation of sulfate as $S^{35}O_4$ in cartilage has

been studied by a number of investigators, foremost among them Bostrom, who isolated chondroitin sulfate from rib cartilage of rats after varying time intervals following the injection of $Na_2S^{35}O_4$ (1952) The maximum uptake was reached in 24 hours and slowly declined to half the maximum on the 17th day. Slices of rib cartilage *in vitro* in presence of O_2 also incorporate sulfate into chondroitin sulfate In boiled slices or under N_2, no radioactivity could be found in the chondroitin sulfate. No information about the rate of synthesis of the carbohydrate chain is available. As stated above, testicular hyaluronidase and presumably other hyaluronidases of animal origin hydrolyse chondroitin sulfate A by opening of the hexosaminidic bonds The oligosaccharide fractions thus produced are sulfated (Linker & Meyer, unpublished). No evidence for the existence of a sulfatase of mammalian origin capable of hydrolysing *in vitro* the ester sulfate group has been obtained. The study of the sulfate esters found in urine should furnish information about this degradation. It seems noteworthy that preparations of chondroitin sulfates contain constantly less than the theoretical amount when calculated on a stoichiometric basis of the CO_2 formed on decarboxylation, the most significant analytical value This finding together with the now established existence of chondroitin makes it probable that the polyacids are first synthesized and then sulfated. It appears rather curious that hyaluronate remains non-sulfated.

It is not known with certainty in what form chondroitin sulfate A occurs in hyaline cartilage Morner already noted that water extracted a small quantity of chondroitin sulfate from minced cartilage (Morner, 1889) Blix (1940) found, by electrophoresis, no evidence for the existence of protein complexes in such aqueous extracts Partridge (1948), who extracted acetone-dried cartilage at elevated temperatures with various aqueous and alkaline solvents, concluded on the basis of analyses and electrophoretic measurements that chondroitin sulfate was partly present as a complex of a non-polar type with collagen Meyer & Smyth (1937), on the basis of the similarity of the ratio of nitrogen to hexosamine in cartilage powder and in salts formed by gelatin and chondroitin sulfate and on the basis of the dissociation of both these complexes by $CaCl_2$, concluded that chondroitin sulfate was present in salt linkage with protein Einbinder & Schubert (1950) found that neutral salts extracted a minimal quantity of chondroitin sulfate from fresh wet cartilage, while the same extractants gave high yields when the wet cartilage was stored at 0° for 4 to 6 weeks From this, they suggest that the binding of chondroitin

sulfate and collagen is other than salt-like and that the linkage may be broken by enzymatic processes

The concentration of chondroitin sulfate (including both A and C) in cattle nasal septa has been reported as about 22 per cent of the dry weight. In tracheal cartilage (pretreated with acid pepsin) the concentration was similar, 25 per cent. How such large quantities are arranged in relation to the collagen fibrils remains an intriguing and formidable problem The behaviour of chondroitin sulfate as a polyelectrolyte has recently been studied by Mathews (1953) He concludes that, in water, chondroitin sulfate exists as an extended chain which coils to varying degrees of tightness depending on the type and concentration of the added cations

3. Chondroitin Sulfate B In 1941, Meyer & Chaffee isolated from pigskin, besides hyaluronate, a substance of the composition of chondroitin sulfate, distinguished from that of hyaline cartilage by its higher optical rotation, approximately —50° as against ~ —30° A fraction with similar rotation and solubilities was isolated from tendon, from heart valve, and aorta (Meyer & Rapport, 1951). From all these tissues, a second chondroitin sulfate was isolated with an $[\alpha]_D$ of —20°. The latter was absent in pigskin From calfskin likewise hyaluronic acid and chondroitin sulfate B were obtained. It is probable that the sulfate fraction of normal and myxoedematous human skin demonstrated by Watson & Pearce (1949) is also chondroitin sulfate B although no conclusive data were presented as to the identity of the sulfated fraction. Bostrom & Gardell isolated chondroitin sulfate, presumably B, from rat skin after injections of $S^{35}O_4$ (1953) The activity of the isolated sulfate showed a maximum 24 hours after the injection. The half-time was reached in 9 to 10 days, thus indicating a more rapid synthesis than of chondroitin sulfate A in costal cartilage. Schiller et al. (1954) reported recently on the isolation of hyaluronic acid and chondroitin sulfate from rabbit skin after injection of carboxy labeled acetate The rate of synthesis of hyaluronate was more rapid than that of chondroitin sulfate. From the decay curve the half-life of the two compounds was calculated as 2, and 7 to 8 days, respectively Chondroitin sulfate A is further distinguished from B by the resistance of the latter to testicular hyaluronidase which hydrolyses chondroitin sulfate A as well as C.

Chondroitin sulfate B is not extracted from the tissues with water or with neutral salt solutions. With 0.02 N alkali, it is obtained as a protein complex which migrates even at pH 8 6 as protein complex The muco-

polysaccharide is dissociated from the protein by treatment with 0 33 to 0 5 N Na OH which was used as an extractive formerly Recently we have liberated *the mucopolysaccharides by combined peptic and tryptic digestion.* No detailed study of the substance has yet been made, aside from the isolation of chondrosamine in yields up to about 80 per cent of the colorimetrically determined quantity, and analytical figures indicating equimolar concentrations of uronic acid, hexosamine, acetyl, and sulfate

4. Chondroitin Sulfate C. One of the fractions isolated from umbilical cord, tendon, heart valves and aorta yielded a chondroitin sulfate which was similar in composition to chondroitin A Like A, it was digested by testicular hyaluronidase and was resistant to pneumococcal hyaluronidase However, the Ca salt had a very much greater solubility in aqueous alcohol It precipitated, in the fractionation procedure used in this laboratory, between 40 and 50 per cent alcohol while A precipitated at 20 to 25 per cent ethanol. It also had a considerably lower optical rotation (—20°) than A (—30°) Later it was found that a fraction corresponding to C also could be isolated from hyaline cartilage, where it represents about one-quarter of the total chondroitin sulfate. Chondroitin sulfate C thus may represent a homologue of chondroitin sulfate A, of lower molecular weight, especially since the polydispersity on sedimentation of the chondroitin sulfate A of cartilage is quite marked (Blumberg & Meyer, unpublished) The amino sugar of the polysaccharide has been shown to be chondrosamine. No other chemical data are at present available From nucleus pulposus, a sulfated fraction has been isolated which contained chondrosamine (Malmgren & Sylvén, 1952) On the basis of its low optical rotation, it may correspond to C With the exception of the cornea, we have never found a fraction with the rotation and solubility of A in umbilical cord or the other mesodermal tissues listed in Table 1, this regardless of the mode of extraction of the tissues

5 Chondroitin. The hyaluronic acid-like fraction isolated from bovine cornea had a rotation of $[\alpha]_D$ —21° and on hydrolysis yielded chondrosamine as the crystalline hydrochloride in a yield of about 75 per cent (Davidson & Meyer, unpublished) The identity of the hexosamine is established by its rotation and the m p. of the 2-hydroxy-naphthylidene derivative (Jolles & Morgan, 1940) Chondroitin thus far has not been isolated from tissue other than cornea Its presence, however, is suggested by the constant and significant deficiency of sulfate in many chondroitin sulfate fractions Chondroitin is hydrolysed by testicular and pneumococcal

hyaluronidase at rates comparable to those of hyaluronate. It is probable that chondroitin is the precursor of the chondroitin sulfates.

6. Keratosulfate. This compound has been isolated only from bovine cornea It is the only mucopolysaccharide of animal origin which does not contain a uronic acid. It is composed of equimolar concentrations of glucosamine, acetyl (as N-acetylglucosamine), galactose and sulfate (Meyer et al., 1953). It represents about 50 per cent of the total mucopolysaccharide fraction of the cornea, the other half is represented in almost equal concentration by chondroitin sulfate and chondroitin. Thus far, no evidence of the existence of keratosulfate in tissues other than cornea has been obtained. The location of the three mucopolysaccharides in the cornea is not known. From the metachromasia of cornea (Jorpes et al., 1937), it can be inferred that they are all derived from the substantia propria.

No enzyme was found which hydrolysed either of the two glycosidic bonds present in keratosulfate The sulfate groups are partially hydrolysed by a sulfatase prepared from the hepatopancreas of the marine snail Littorina (Davidson & Meyer, unpublished).

Analysis of Function of the Ground Substances

It is evident from the foregoing that the areas of ignorance of the chemical composition of the ground substances are wide indeed. The analysis of their function, however, is almost entirely unexplored This section will enumerate a few of the probable functions of the ground substances. Hyaluronic acid appears to function in water binding, as a lubricant and as a shock absorber The structure of hyaluronic acid, as a highly hydrated coiled sphere easily deformed by flow and easily "drained", appears to explain well these biological functions. The chondroitin sulfates and other new sulfated polysaccharides lack these properties and, in fact, occur in tissues which are normally in a state of dehydration. They probably are in intimate contact with the fibrous elements and actually may form part of the fibrous structures They have early been connected with biological fibrinogenesis and with the binding of cations In fact, they have been demonstrated to function analogous to synthetic ion exchange resins (Boyd & Neuman, 1951).

In vitro fibrillogenesis has been studied by electron microscopy of extracted collagen or the so-called procollagen (Orekhovich et al., 1948).

A variety of substances including neutral mucoids and acid polysaccharides have been shown in this work to "reconstitute" collagen fibrils of varying periodicity (Schmitt et al, 1953). That this reconstitution bears any relation to biological fibrillogenesis, however, is not very probable. The biological fibrillogenesis, a priori, can be expected to be principally a question of extracellular polymerization, and there is no evidence that this mechanism depends on the presence of factors active in the *in vitro* reconstitution.

Other biological interactions, such as calcification and the avascularity of hyaline cartilage and cornea, have been associated with the sulfated mucopolysecchandes. While some experimental evidence can be adduced implicating the former, too little is known about the properties and the metabolism of the different mucopolysaccharides to merit a discussion of the processes

The interaction of acid mucopolysaccharides with protein and with dyes has received considerable attention, the first in connection with the nature of the protein complexes which occur or are supposed to occur in the tissues, the latter in connection with the staining reactions of the tissues. The protein-mucopolysaccharide interactions have been discussed above (see Faraday Soc 1952). The interaction of mucopolysaccharides with dyes still remains a controversial field (For a very competent discussion, see Persson, 1953) It is now generally agreed that hyaluronate as well as the sulfated mucopolysaccharides will stain metachromatically Two main problems have to be dealt with · (1) the nature of the molecular change of the dye, and (2) the nature of the compounds inducing the change from ortho- to metachromasia The first problem is still not solved (Schubert & Levine, 1943) The second problem appears to be much clearer, especially on the basis of the fundamental work of Bank & Bungenberg de Jong (1939) These authors showed that the basic dyes reacted with the polyanions in a stoichiometric relation. Two types of bonds were involved, polar and van der Waals forces, leading first to "electroadsorption" of the dye to the anionic colloid and secondarily to an aggregation of the dye molecules In the staining reaction, the dye cation has to compete with the cations of the tissue, especially protein, for the polyacids. The *primary* reaction of the dye in the tissue is similar to ion exchange reactions and the previous treatment of the fluid or tissue beside the nature and quantity of the polyacids and polybases will determine whether and to what extent metachromasia is displayed The diagnostic value of the reaction is thus quite limited as are other staining reactions

Conclusions

The preceding pages represent a very incomplete review of our knowledge of the chemistry of the ground substances. This review deals almost exclusively with mucopolysaccharides because (1) these substances are the only components about which we have definitive information and (2) they appear to be the most typical for connective tissue. It is to be expected that future research will continue on these components in a predominantly analytical approach It is to be hoped that histological methods will be perfected which localize the fractions isolated chemically and that the all-important biological interrelations between cellular and fibrous elements and the ground substances and the relation of connective tissue to parenchyma and endothelium can be tackled on a firm experimental and theoretical basis. Only then may we expect an understanding of the pathological mechanisms in which the connective tissue are primarily involved

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RECENT ADVANCES IN *THE CHEMISTRY OF COLLAGEN*

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Introduction

THE REASON FOR INCLUDING this chapter is the present dynamic state of the chemistry of collagen New aspects of its structure, metabolism and fibre formation are emerging, and medical applications can be expected in the near future Collagen has been extensively reviewed from the physical and chemical standpoints by Bear (1952). The most comprehensive single source of up-to-date information is a symposium report, edited by Randall (1953). The latter especially contains much biologically relevant material.

The present subject matter is divided into two parts, one dealing with the insoluble collagen and the other with soluble collagens and their metabolic behaviour and fibrogenesis.

Insoluble Collagen

In the last decade X-ray diffraction studies and electron microscopy have revealed new morphological criteria for collagen as a structural unit and thus have supplied a basis for the molecular models The composition of collagen is now well known and the knowledge of the arrangement of amino acids in the polypeptide chain is progressing Reticulin is accepted into the collagen group but its nature is still a matter of dispute.

X-RAY DIFFRACTION

High-angle X-ray diffraction has provided a valuable tool for obtaining information on the submicroscopic structure of the polypeptide chain and the data (the reader is referred to Bear or Randall) show a residue repeat at 2.86 Å intervals and a spacing in the width ranging from 10 4 to 16 Å depending on humidity Two kinds of molecular models have been

proposed, one composed of sheets made up of parallel chains, hydrogen bonded to layers of 10 4 Å distance and separated by swelling. The other model, which better accounts for the density and crystallographic theory, presupposes a helical structure, and in the original presentation three of the helices were hexagonally packed together to form a fibril (Pauling & Corey, 1951) Bear's model consists of a single helix with the 20 Å unit of 7 nearly equivalent groups of three residues each (Bear, 1953). The final form has obviously not yet been reached (cf. Randall, 1953, p. 241). The amino acid sequence also must be substantially known and taken into consideration

The small-angle X-ray diffraction studies have also been successful, and the 640 Å repeat, found previously by electron microscopy, has been confirmed and the nature of the interbands investigated (summary by Bear, 1951)

Electron Microscopy

Classical histology has not attained much progress with its empirical methods. The fibres seen with the light microscope are actually thick bundles of fibrils and electron microscopy is needed to resolve them. The pattern, first revealed by Schmitt et al (1942) and Wolpers (1943), has provided a new definition for the collagen group of scleroproteins It should be pointed out, however, that the chemical composition may differ considerably, for example, in proline content, which generally is held to be essential in the collagen composition (cf. Bear, 1952; Randall, 1953, p 232).

The typical collagen banding consists of main bands at intervals of 640 Å with an asymmetrical subspacing where as many as 18 bands have been resolved Two other forms of spacing have been obtained. The embryonal and newly formed collagens on the surfaces of the cultured fibroblasts show a main pattern of 210 Å spacing (Porter, 1951; Randall et al, 1952) In the precipitated forms of collagen (cf. *infra*) a "long spacing" (1800–3000 Å) has been noted but is not found in nature Their subbanding is occasionally symmetric. These long-spaced and the normally spaced fibrils are interconvertible by varying the ionic strength (Schmitt et al, 1953)

There are two main concepts of the chemical basis of the banding. The first would explain it by the mere folding of the chain by hydrogen bonding; according to the other, the arrangement of the bands is deter-

mined by the distribution of the amino acid sequences. The polypeptide chains tend to match their common chemical features. The collagen fibril would consist alternatively of perfectly and imperfectly matching regions, the "electronic stain" in the latter penetrating more easily. The lysine side chain, it is suggested, occurs frequently at the band levels (Bear, 1951). The convertibility of the spacing seems to imply, at least some significance to the folding

Composition of Collagen

The amino acid composition of collagen is very well documented and the analysis by Bowes & Kenten (1948) is generally accepted. The typical amino acid composition, namely the preponderance of glycine and both prolines, the presence of hydroxylysine and the paucity of sulphur-containing and aromatic amino acids, forms the chemical definition of collagen The values for hydroxyproline, which can be determined specifically, have been used to indicate the total content of collagen.

The reactivity of the side chain groups in the polypeptide chain has been extensively studied by Bowes and her colleagues All the carboxyl groups are esterifiable The availability of the free α- and ε-amino groups has been studied using the condensation with dinitrofluorobenzene. The insoluble collagen yields no free terminal amino groups and only about one-half of the total ε-amino groups of lysine are reactive. Gelatin and modified collagens yield small amounts of N-terminal amino acids (mostly aspartic acid, alanine and glycine) as shown by Bowes & Moss (1953) and Grassmann & Hormann (1953) Heyns & Konigsdorf (1953) observed after a short hydrolysis a temporary rise in the reactivity of the ε-amino groups All the ε-amino groups could be acetylated in a mildly alkaline medium but only 77 per cent of the hydroxyl groups (Green et al., 1953).

The arrangement of the amino acids in the polypeptide chain has been actively studied since Bergmann, who postulated the sequence X-P-G-X, where G stands for glycine, P for either proline or hydroxyproline and X for some other amino acid The peptide -lys-pro-gly- (conventional abbrevations) was isolated already in 1936 by Grassmann & Riederle, but not until the availability of chromatography and DNP-technique was more extensive work possible. I have here the privilege to use Schroeder's communication (1953) on the isolation of 34 peptides from partial acid and basic hydrolysates of gelatin. About 20–30 per cent of glycine, proline, alanine

TABLE 1

Composition of Collagen and Citrate Extracted Collagen

Material	Collagen g/100 g	Citrate extracted collagen g 100 g
Total nitrogen	18 60	17 70
Amino nitrogen	0 46	0 49
Glycine	26 2	26 07
Alanine	9 5	9 95
Leucine	5 6	3 20
*Iso*leucine		1 39
Valine	3 4	2 26
Serine	3 4	4 23
Threonine	2 4	2 21
Methionine	0 8	0 78
Cystine	0 0	0 0
Proline	15 1	13 02
Hydroxyproline	12 83	13 62
Phenylalanine	2 5	1 99
Tyrosine	1 4	0 50
Tryptophan	0 0	—
Arginine	8 8	8 34
Histidine	0 8	0 29
Hydroxylysine	1 3	0 90
Unidentified[1]		0 38
Lysine	4 5	3 57
Aspartic acid	6 3	6 05
Glutamic acid	11 3	11 02
Amide nitrogen	0 66	0 52
Hexosamine	0 33	0 19

[1] probably *allo*-hydroxylysine

This table is reprinted from the article by *Bowes, Elliott & Moss* in *Nature and Structure of Collagen* (Butterworth) by the authors' and publisher's kind permission The columns showing the values as amino acid nitrogen in percent of total protein nitrogen are omitted The author is especially indebted to Dr J H Bowes, who allowed the use of the corrected values of the citrate extracted collagen which are in the course of publication

and serine, 13 per cent of hydroxyproline and 42 per cent of threonine were accounted for as peptides The most common were the following in the order of decreasing quantity gly-pro, hypro-gly, thr-gly, ser-gly, ala-gly, gly-ala, gly-glu, glu-ala, ala-ala and glu-gly The first two were by far the most common The nature and quantity of the peptides do not support the above-mentioned "periodicity theory", but the peptide sequence -gly-pro-hypro-gly- (or a similar sequence) may occur frequently in gelatin

The nature and amount of the carbohydrate moiety of collagen is not

confirmed by a generally accepted analysis although its occurrence has been proposed by many authors Grassmann et al. (1937) concluded that collagen contains hexosamine, galactose and glucose (about 0.6 per cent together). A carbohydrate containing in addition mannose has been assumed to form a part of the collagen fibrils (Gross et al., 1952). The question is complicated by the fact that in the anatomic environment—perhaps also as the chemical complex—there exist in the ground substance carbohydrates containing all the mentioned sugars (Consden, 1953).

Reticulin

The definition of reticulin is essentially morphological and it is now well established that it gives the collagen spacing and has also a similar amino acid composition (Little & Kramer, 1952, 1953). Reticulin does not yield gelatin by boiling The relation of reticulin and collagen is according to the cited authors similar as that between rope and linoleum. The fibrous material is the same, but in reticulin it lies embedded in the amorphous matrix presumably consisting of polysaccharides Reticulin is claimed to possess antigenic properties (Cruickshank & Hill, 1953).

Soluble Collagens

Soluble modifications of collagen have been known since 1900 but the work by the Russians on the preparation of "procollagen" has stimulated wider interest (cf review in French by Orekhovich, 1952). The molecular properties of soluble collagen are the subjects of studies still in progress The aggregation and precipitation of the dissolved particles to fibrils is of almost paramount interest from the medical point of view. The metabolic aspects have been elucidated by various isotope methods and collagen has been added to the biologically active proteins. The pathology involving these collagens is opening new aspects but the data accumulated are thus far very meagre

Preparation

The solubility of the rat tail tendon in dilute acetic acid was studied by Nageotte (1927) and he already found that the soluble collagen could be recovered even in crystalline form differing herein from gelatin and

other degraded collagens. The use of organic acids was extended by the Russian group (cf. Orekhovich, 1952) since 1947.

The Russian workers pretreated the skin with about 0.2 *M* disodium phosphate to remove the "albumins and globulins". Later Highberger et al. (1951) isolated from this extract an additional collagen fraction by dialysis against citrate at pH 3.8. It had approximatively the chemical composition of collagen and in the electron microscope the collagen spacing. This fraction is referred as alkali-soluble extracted collagen

The best sources of the soluble collagens are mammalian and fish skin, tendons and the fish swim bladder tunic. Quantitatively most important is the citrate-extracted collagen and it is obtained by extracting the alkali-treated skin residue with 0.1–0.2 *M* sodium citrate buffer at pH 3.0–5.7 (depending on the species) in the low temperature (about +8° C or less). The solution is highly viscous The protein is recovered either by dialysing the buffer away or by adding neutral salts In many cases the precipitate is amorphous but occasionally crystallises in needles The amounts differ in various tissues The alkali-soluble collagen contained one per cent of the total nitrogen, the acid soluble six per cent, and the insoluble collagen 30 per cent in a rabbit skin sample (Harkness et al, 1953) The Russian authors obtained about 1–2 per cent citrate-extracted collagen from the dry weight of rabbit skin

The solubility of collagen is enhanced by many organic acids especially by those containing two or more carboxyls The solubility is rather low in the physiological salt concentration and pH but is enhanced also there by citrate and calcium ions in their physiological concentrations (Kulonen et al, 1953) If a saturated solution is brought from the ice-box to room temperature and warmed, the viscous solution turns gelatinous and turbid, shrinks and forms a fibrous mass

It is not possible to state at present whether the protein originates from the ground substance or from the fibres. Morphological study of the residue shows that the fibres are swollen, spindle-shaped and the fibrils dispersed When the tissue was extracted six times successively for 24 hours some matter was still obtained The fractions do not differ in the ultraviolet absorption spectrum or in the intrinsic viscosity. The Russian workers claim that in the successive fractions the tyrosine content increases. There is also evidence which is interpreted to show that insoluble part of the fibres contains relatively more tyrosine and hexosamine but less hydroxyproline (Bowes et al, 1953).

Molecular Properties

The molecular weight of the soluble collagen has been studied by many authors. Bresler et al (1950) combined ultracentrifugation and diffusion data and calculated a molecular weight of about 65 000. The same order of magnitude was obtained by osmotic pressure measurements (Mathews et al., 1954) In striking contrast to these are the values obtained by light-scattering (M'Ewen & Pratt, 1953; Mathews et al, 1954) and end-group (Bowes & Moss, 1953; Kulonen, 1954) methods Both give values of about one million and more. The most plausible explanation might be that there exist in solution aggregates of differing sizes. The methods used are selectively sensitive to the aggregates of a particular weight.

There is evidence obtained by ultracentrifugation (Bresler et al, 1950) and by electrophoresis (Brown & Kelly, 1953) that aggregates (side to side and end to end) are formed after addition of salts The isoelectric point of collagen is about pH 5 8 and the mobility is in the same order as that of gelatin.

To obtain information of the molecular form, studies have been conducted on the viscosity characteristics The specific and the intrinsic viscosities are very high (about 15) and the axial ratio can thus be calculated to be about 200 1 Bresler et al deduced a much lower axial ratio (23:1) and a molecular length of 380 Å The high axial ratio is not in agreement with low molecular weight values Studies have also been made on the viscosity in regard to the velocity gradient, and it has been demonstrated that the collagen forms a real Newtonian solution (Mathews et al, 1954).

The viscosity is the highest found in the solutions of the animal body constituents The viscosity can be reduced to the level of the serum proteins by adding urea or by heating for a short time (Mathews et al, 1954). These reversible phenomena indicate that the high viscosity may be maintained by hydrogen bonding, which is believed to give also to the insoluble collagen its mechanical stability. The reactivity of the soluble collagen with dinitrofluorobenzene in both dissolved and precipitated forms is similar to that of the gelatin (Kulonen, 1954)

Fibrogenesis

The possibility of producing collagenous fibrils *in vitro* has aroused much interest owing to the apparent biological implications. The question also seems to involve the relationship of the ground substance polysac-

charides to the fibres and their formation When salt is added and the pH simultaneously raised by dialysis a normally spaced collagen fibril is obtained. When certain additives are used either in connection with salt or alone, fibre formation is also observed but with the "long-spacing". The mechanism of these reactions is not clear Many additives have been studied, including acid mucopolysaccharides, heparin, serum glycoproteins, other protein fractions and plant gums (Highberger et al, 1951, Gross et al, 1952, S F Jackson & Randall, 1953) Owing to the wide variety of the precipitating agents the fiber formation seems to have been non-specific in these experiments Under particular conditions a segmented long spacing has been observed (Randall, 1953, p 220) and similar segments are also obtained by the addition of adenosine triphosphate (Schmitt et al, 1953), which forms a part of the precipitate The state of aggregation of collagen is thus determined by the presence of macromolecular substances in co-operation with small ions and pH. The much discussed problem of intra- or extracellular fibrogenesis has been studied by Porter (1951) and by S F Jackson (1953) At least the last stage seems to occur at the cell surface

In this connection two special observations deserve to be noted D. S Jackson (1953) demonstrated that the tendon collagen is actually more soluble if the tissue is pretreated with testicular hyaluronidase This supports the view of the crosslinked structure of connective tissue (Partridge, 1948). The electron micrographs show that dissolution of the collagen fibril can occur in corpuscular particles (Randall, 1953, p 235) On the other hand, the action of collagenase does not dissolve the segments separately but a tapering is observed in the end of the fibrils with a preserved spacing (Gross, 1953)

Metabolic Aspects

The Russian workers use the term "procollagen" to indicate the belief that the citrate-extracted collagen is the biological precursor of insoluble collagen They produced isotopic data to support this thesis (cf Orekhovich, 1952) Neuberger with collaborators (1951, 1953) has followed the utilisation of labelled glycine to the collagen fractions in the rat tissues The results show that the turnover of the insoluble collagen is very low, being fastest in young animals and in bone collagen. With special reference to the soluble collagens they found that the alkali-soluble collagen of

the rabbit skin is almost certainly the precursor of all or most of the insoluble collagen but the citrate-extracted can be the precursor for only a part of the insoluble form (Harkness et al., 1953). When labelled acetate was injected into a rabbit and the successive citrate extracts were later prepared, the total activity was, as expected, in a decreasing order and the distribution in the different amino acids was in agreement with the studies on the intracellular proteins, *i e.*, that most of the activity was in aspartic and glutamic acids and in prolines (Kulonen, 1954).

No pathological X-ray diffraction patterns, electron microscopic spacings or molecular forms are known. The disintegration of the fibrous collagen to a structureless mass has been described with electron micrographs. Subcutaneous rheumatic and fibrinoid nodules have been studied (Consden et al., 1953) and data on collagen constituents are also presented. In the collagen in a benign fibroma the normal birefringence disappeared (Ambrose in Randall, 1953, p. 223). The biology of nucleus pulposus has been studied with special reference to the collagen and polysaccharides (summarising report by Sylvén, 1951; Happey et al., 1953). With advancing age the collagen tends to crystallise from the original gel structure

The amount of the soluble collagen seems to decrease with the age (from 7–10 per cent to 1–2 per cent in the rabbit skin) Studies are also available on the collagen fractions in the ascorbic acid deficiency (Orekhovich, 1952). The amount of the alkali-soluble collagen, containing a relatively small amount of hydroxyproline, seems to have been increased in the deficient state (Robertson & Schwartz, 1953). In thyroidectomised rats the insoluble skin collagen content was subnormal but the injection of the growth hormone increased it considerably (Scow, 1951).

The number of biological applications is meagre but the basic knowledge accumulated is stimulating This field has long suffered from the lack of suitable methods, even if the significance of the connective tissue pathology has been well established. The great problems of the fibrogenesis during growth and regeneration now have chemical lines of approach and also the physiology and pathology of the ground substance can be better understood.

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METABOLISM OF THE MUCOPOLYSACCHARIDES OF CONNECTIVE TISSUE

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Introduction

THE TERM acid mucopolysaccharides has been widely used to denote a group of chemical compounds which appear to be high molecular weight polymers composed of the hexosamines, glucosamine or galactosamine, glucuronic acid, acetate and esterified sulfate To date, two of these substances, hyaluronic acid (H.A.) and chondroitin sulfuric acid (C.S A) have been well characterized in connective tissue Although extensively investigated, the chemical nature of heparin is still the subject of some confusion, with evidence that there may be a family of such compounds There appears also to be evidence of the existence of a compound whose composition is identical with that of C S A. of cartilage but differing in other properties Thus Meyer & Chaffee (1941) and Meyer & Rapport (1951) have suggested that chondroitin B, which exists in skin, differs from cartilage chondroitin (A) in its optical rotation and susceptibility to hydrolysis by testicular hyaluronidase (cf p 64). What appears to be a similar compound has been isolated by Marbet & Winterstein (1951, 1952) from lung and originally called β-heparin because of anticoagulant properties. A possibly identical substance has been isolated by Smith & Gallop (1953) from hog gastric mucosa (polysaccharide B) In recent studies Schiller, Mathews & Dorfman (1954) have isolated, in analytically pure form, a sulfated polysaccharide which appears to be identical

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with cartilage chondroitin in composition, but gives a smaller color equivalent by the Dische carbazole reaction. Treatment of this substance with testicular hyaluronidase results in some apparent hydrolysis as measured by the turbidity method, suggesting this may be a mixture of cartilage C.S A and so-called chondroitin B or β-heparin. A fifth acid mucopolysaccharide, named chondroitin C, has also been postulated by Meyer & Rapport (1951).

In the last few years there has appeared extensive biological and medical literature regarding the ground substance of connective tissue. Unfortunately the term ground substance has been used loosely, some authors considering "ground substance" as a uniform chemical substance, while others *consider the term ground substance synonymous with extracellular fluid.* With this confusion of definition, extensive theories regarding the behavior of connective tissues, mechanisms of action of hormones, etc., have been propounded on the basis of evidence obtained by the use of histochemical techniques which have not been validated

In order to clarify our understanding of the nature and function of ground substance some fundamental agreement regarding definitions seems desirable Dorfman (1953) has proposed that the term ground substance be reserved for the amorphous continuum separating the cells, fibers and vessels of connective tissue This solution is made up of a variety of components, some of which may be regarded as peculiar to the connective tissue, while others may be regarded as in transit between the circulation and the parenchymal cells. The acid mucapolysaccharides appear to be peculiar to the connective tissue.

The fact that these substances are flexible chain polymers of high negative charge with a high affinity for cations and water molecules suggests that they may play a critical role in regulating the metabolism of inorganic ions and water A change in concentration or molecular size of such a substance is obviously of great importance in modifying the capacity of connective tissue to bind water and salts. It is thus possible that the ground substance may act as a selective and controlled barrier between the circulation and parenchymal cells

Recent evidence has indicated that a soluble precursor of collagen is *present in the ground substance* (Schmitt et al, 1953). The possible interactions of the acid mucopolysaccharides with soluble collagen requires further elucidation. Jackson (1954) has suggested that chondroitin plays a significant role in the structure of collagen fibers in tendon.

Although a somewhat detailed understanding of the enzymatic mechanism of synthesis of homologous polysaccharides such as starch or glycogen has been achieved, little is known regarding heterologous polysaccharides to which group belong chondroitin sulfuric acid (C.S.A.) and hyaluronic acid (H.A.).

It is the purpose of this chapter to try to bring together available data concerning the metabolism of the mucopolysaccharides. The first sections will be concerned with the metabolism of hexosamines and glucoronic acid. No attempt will be made to discuss the hydrolysis of hyaluronic acid by the enzyme hyaluronidase and only limited consideration will be given to metabolic studies utilizing radioactive sulfate in view of discussions elsewhere in this book.

Metabolism of Components

Glucuronic Acid

Studies of the metabolism of this compound have been numerous, in view of its importance in the process of detoxification The literature along these lines has been extensively reviewed by Artz & Osman (1950) Certain fundamental metabolic questions regarding glucuronic acid, although the subject of numerous investigations, remain of importance These can be stated as follows.

1) What is the precursor of glucuronic acid and by what mechanism does conversion to glucuronic acid occur?
2) Does glucuronide synthesis involve the utilization of glucuronic acid or does some other glucoside intermediate form before oxidation of the C-6 of the carbon chain?
3) What is the relationship of β-glucuronidase to synthesis of glucuronides?
4) What is the relationship of glucuronic acid formed in the liver for detoxification, to connective tissue polysaccharides containing glucuronic acid?
5) What is the relationship of glucuronic acid to ascorbic acid synthesis?

While space does not permit a detailed consideration of these problems, certain definitive information is available pertinent to these questions

Despite the extensive discussions of the older literature and the more recent claims (Bidder, 1952, Doerschuk, 1952) of the role of three carbon particles as precursors of glucuronic acid, recent tracer studies conclusively demonstrate the derivation of glucuronic acid from glucose without previous scission of the glucose molecule (Mosbach & King, 1950; Douglas & King, 1953 a; Eisenberg & Gurin, 1952, Roseman et al, 1954).

The presence of glucuronic acid in the mucopolysaccharides of connective tissue has led some (Peterman, 1947) to consider the possibility that metabolism of this compound may be related to diseases of connective tissue. On the basis of this concept, glucuronolactone has been suggested (Hodas *et al*, 1949) as *a therapeutic agent presumably particularly useful* in the treatment of degenerative arthridites. No adequately controlled study has so far appeared demonstrating the validity of such suggestions.

The question of whether glucuronic acid is first formed and then linked to phenols or whether oxidation of the C-6 carbon atom occurs after glycoside formation has been investigated for many years by various techniques with no final answer yet at hand. Packham and Butler (1952) have compared the incorporation of C^{14} into α-naphthol glucuronide in rabbits after the administration of labeled lactate, pyruvate, glucose and glucuronic acid Since intraperitoneal administration of labeled glucuronic acid resulted in greater incorporation of isotope it was concluded that administered glucuronic acid may be utilized for glucuronide synthesis Somewhat similar results were obtained by Douglas & King (1952) following the administration of glucuronic acid to guinea pigs receiving borneol. Only a small amount of the radioactivity of the glucuronic acid was recovered in the urinary glucuronide. Some doubt arose whether this represented utilization of the glucuronic acid since the isotope distribution of the excreted glucuronide differed from that of the administered glucuronide, suggesting asymmetric resynthesis from 3 carbon particles. Evidence of such asymmetric synthesis was found by Bidder (1952) & Doerschuk (1952) and has recently been demonstrated for the synthesis of glucose from glycerol by Schambye & Wood (1954) and Swick & Nakao (1954). In a later investigation Douglas & King (1953 b) have reinvestigated this question in the guinea pig and the albino rat, utilizing 6-C^{14}-glucuronic acid. Glucuronic acid was rapidly metabolized A small amount of radioactivity was present in the excreted glucuronide which on degradation again showed concentration of radioactivity in the first 3 carbon atoms. It was of particular interest in this study to note that the C_6 of glucose isolated

from the glycogen was quite active, suggesting the possibility that glucuronic acid may be converted to glucose.

Further understanding of the mechanism of glucuronide synthesis has recently been suggested by the publications of Storey (1950) & Dutton & Storey (1951, 1953), reporting that liver contains a thermostable factor necessary for the synthesis of o-aminophenol glucuronide by liver homogenates. Preliminary evidence suggests that this factor contains uridine diphosphate and glucuronic acid, a finding of great interest in view of the recent discovery of several uridine diphosphate compounds containing different hexoses (Caputto et al., 1950, Leloir et al, 1951; Park, 1952a, b; Paladini & Leloir, 1951).

The role of the enzyme β-glucuronidase has attracted a great deal of attention. This work has been extensively reviewed by Fishman (1950) and questions regarding its possible relationship to biosynthesis of glucuronides and significance of variation in its level in tissues are discussed in some detail by that author. From the point of view of metabolism of mucopolysaccharides Meyer et al (1951) have presented evidence that β-glucuronidase serves to degrade to monosaccharides, the disaccharide which is formed as a result of the action of testicular hyaluronidase on hyaluronic acid. What role that action has under physiological conditions is not as yet clear

Although it is apparent that glucuronic acid plays a major role in the process of detoxification and in the structure of acid mucopolysaccharides, the interrelationship between these two functions has not been explored. A number of studies have demonstrated that glucuronide synthesis probably occurs in the liver while it seems most likely that mucopolysaccharide formation occurs as a result of the metabolic activity of connective tissue cells (fibroblasts, mast cells) In the studies of Douglas & King (1953 a) little radioactivity of exogenous glucuronic acid was fixed in tissues.

The relationship of ascorbic acid to connective tissue is of considerable interest and importance It is beyond the scope of this review to detail all pertinent observations along these lines, but it is of some importance to point out the biochemical relationships between glucuronic acid and ascorbic acid On the basis of studies utilizing C^{14} labeled glucuronic acid, Horowitz & King (1953) concluded that glucuronic acid is probably an intermediate in the synthesis of ascorbic acid from glucose in the rat.

Such a mechanism has received striking support from the recent studies of Isherwood, Chen & Mapson (1954) who found that of many related

carbohydrate derivatives only L-gulono, L-galactono- and D-glucurono-γ-lactones and D-galacturonic acid methyl ester were converted to ascorbic acid by cress seedlings and rats In a subsequent study Mapson, Isherwood & Chen (1954) demonstrated the *in vitro* conversion of L-galactono-γ-lactone to ascorbic acid by mitochondria obtained from pea seedlings.

Hexosamine

In addition to their well established presence in the mucopolysaccharides, the hexosamines are found in varying amounts in a large number of proteins, being at particularly high levels in the mucoproteins.

Recent studies utilizing isotopic tracer techniques have made it quite clear that glucosamine derives from glucose without previous scission of the carbon chain of glucose in at least several sources. Thus, studies by *Roseman et al (1953, 1954) have demonstrated that the incorporation of* 1-C^{14}-glucose or 6-C^{14}-glucose into a semi-synthetic medium upon which a strain of Group A streptococcus is grown, leads to the formation of H A., the glucosamine of which contains C^{14} of the same activity (in the appropriate carbon atom) as the starting glucose. Identical results were obtained by Topper & Lipton (1953) utilizing the same organism. Likewise, the studies of Becker & Day (1953), with regard to the formation of glucosamine in serum proteins of the rat resulted in similar conclusions. Studies by Rieder (1953) of the synthesis of the glucosamine of the egg proteins (principally in the ovomucoid and ovomucin) also indicate the utilization of the intact glucose molecule for the synthesis of glucosamine These authors found that glycine (labeled with N^{15} and C^{14}) did not serve as a glucosamine precursor

The mechanism of amination has not been entirely clarified Lowther & Rogers (1953), in a preliminary report, found that glutamine or glutamic acid plus ammonia were necessary for the synthesis of H.A. by a resting cell suspension of hemolytic streptococci, indicating that the amide group of glutamine serves as the precursor of the amino group of glucosamine. Leloir & Cardini (1953 a) have studied the mechanism of amination in extracts of *Neurospora crassa* as part of a study of the mechanism of synthesis of chitin Evidence was found that glutamine is necessary for amination; the reaction presumably being between hexose-6-phosphate and glutamine to yield glucosamine-6-phosphate.

Becker & Day (1953) have proposed that glucosone serves as inter-

mediate in the conversion of glucose to glucosamine. This conclusion was reached on the basis of the finding that the administration of 1-C^{14}-glucosone to rats resulted in the incorporation of a greater amount of C^{14} into glucosamine isolated from the serum proteins than was obtained as a result of the administration of an equivalent amount of 1-C^{14}-glucose. A similar conclusion was reached by Topper & Lipton (1953). Dorfman et al. (unpublished results) have reinvestigated this question and found that 1-C^{14}-glucosone is a relatively inefficient precursor of glucosamine. It was postulated that the apparent conversion of glucosone to glucosamine may result from the prior conversion of glucosone to glucose In order to test this hypothesis, Dorfman et al (unpublished results) utilized uniformly labeled glucosone in experiments with streptococci. It had previously been demonstrated that glucose serves not only as a precursor of glucosamine, but also as a precursor of the glucuronic acid portion of the H A. molecule. If glucosone is incorporated *via* glucose, the glucuronic acid should be labeled to the same extent as the glucosamine This was found to be the case

Detailed information regarding the enzymes responsible for the degradation of glucosamine and N-acetyl glucosamine in mammalian tissue is as yet lacking A number of older studies suggest that glucosamine may be deaminated in the liver to yield glucose (Grafe & Meythaler, 1928; Drury & Salter, 1928, Salter et al, 1935, Yositake, 1939). Other older studies on the degradation of glucosamine both *in vitro* and *in vivo* are reviewed by Kawabe (1934 b) who also conducted studies of both the *in vivo* and *in vitro* degradation of glucosamine (Kawabe, 1934 a). Although evidence of degradation of glucosamine was obtained, the methods used and the results obtained permit no more quantitative or detailed conclusion In a series of similar studies Watanabe (1936) studied the metabolism of N-acetyl glucosamine both *in vivo* and *in vitro* With *in vivo* techniques they found little evidence of N-acetyl glucosamine utilization, although evidence of the existence of a deacetylase in liver was found *in vitro*

In a more recent study performed with considerably better enzyme techniques, Lutwak-Mann (1941) observed that the addition of amino sugars resulted in an increase in oxygen uptake and ammonia production by renal cortex, testis, brain cortex, ovaries, retina and to a lesser extent lung tissue Liver, intestine, mucus membrane, cartilage, spleen, erythrocytes, plasma and synovial fluid had no such activity. Glucosamine, galacto-

samine and N-acetyl glucosamine were all utilized. The oxygen uptake in animal tissues (rat and rabbit) is accompanied by ammonia liberation and acid formation but no liberation of CO_2. In yeast and certain bacteria (*B. coli, Streptococcus faecalis,* and *Proteus vulgaris*) evidence of similar metabolism was found, but unlike the mammalian tissues this proceeded anaerobically as well as aerobically The liberation of acid corresponded to the oxygen uptake, but not to ammonia production, suggesting that oxidation may occur before deamination.

Deacetylation of N-acetyl glucosamine in bacteria was suggested by the finding of a volatile acid in the medium following the action of a variety of strains of streptococci on N-acetyl glucosamine. Direct confirmation of this is furnished by the demonstration of radioactivity in the acetic acid of the medium after a strain of Group A streptococcus has been grown in carboxyl labeled N-acetyl glucosamine (Dorfman et al., unpublished results) A cell free preparation of such a deacetylase has been obtained from *B. coli* (Roseman, 1954).

Harpur & Quastel (1949) found evidence of phosphorylation of glucose, fructose, and glucosamine by an acetone powder of white matter of brain. Since the phosphorylation of the three hexoses was not additive it was concluded that a common enzyme was responsible. The rate of reaction was considerably slower for glucosamine than for glucose and fructose. N-acetyl glucosamine was not phosphorylated and appeared to act as an inhibitor (apparently competitive in nature) of the phosphorylation of the glucosamine, glucose and fructose. Further evidence of the phosphorylation of glucosamine was obtained by Grant & Long (1950) who showed that yeast hexokinase in the presence of adenosine triphosphate, magnesium chloride, potassium fluoride and potassium phosphate catalyzed the formation from glucosamine of a substance, the analyses of which suggested it was a monophosphoglucosamine. Brown (1951) studied this phosphorylation in somewhat greater detail, utilizing crystalline yeast hexokinase and succeeded in identifying the product of the phosphorylation as glucosamine-6-phosphate. Subsequent studies by Brown (1953) showed phosphoglucomutase obtained from rabbit muscle catalyzed the conversion of glucosamine-6-phosphate to a compound with properties consistent with a structure of glucosamine-1-phosphate. The rate of mutation of the glucosamine ester was found to be very much slower than that resulting from the action of phosphoglucomutase on the corresponding glucose ester.

A somewhat different pathway of formation of glucosamine phosphate esters has been proposed by Leloir and Cardini (1953 a). These authors demonstrated amination of glucose-6-phosphate to form a compound which was thought to be glucosamine-6-phosphate. It was proposed that mutation to the 1-phosphate ester occurs following acetylation although details of this mutation have not yet been published, but it is said to be accelerated by glucose-1,6-diphosphate

Relatively little information is as yet available regarding the metabolism of galactosamine although Cardini & Leloir (1953) have recently demonstrated its phosphorylation in the presence of adenosine triphosphate and liver and brain extracts as well as a yeast enzyme The enzyme activity appeared to be parallel to the galactokinase activity The product formed, although not positively identified, appeared to be galactosamine-1-phosphate

The amino sugars are usually found to be acetylated An exception appears to exist in the case of heparin where the amino group is thought to be sulfonated (Wolfrom & Montgomery, 1950; Wolfrom et al., 1950) Chou & Soodak (1952) showed that glucosamine and chondrosamine (galactosamine) can be acetylated by an enzyme prepared from pigeon liver. In crude extracts acetylation was demonstrated in the presence of adenosine triphosphate, co-enzyme A and acetate, while in purified preparations acetyl phosphate and transacetylase were used as the acetyl donor system It was suggested that the glucosamine acetylating enzyme differs from that involved in the acetylation of arylamines

Leloir & Cardini (1953 a) found evidence of acetylation by a similar system with an enzyme obtained from *Neurospora crassa*, but in this case acetylation apparently also occurs with glucosamine-6-phosphate as a substrate It is not as yet clear whether physiological synthesis of polysaccharides involves acetylation before or after phosphorylation. Leloir & Cardini favor the idea that acetylation occurs after phosphorylation in view of the fact that they were unable to demonstrate phosphorylation of N-acetyl glucosamine This view is in keeping with the finding by Dorfman et al. (unpublished results) that no evidence could be obtained for the direct utilization of N-acetyl glucosamine in the synthesis of H A by hemolytic streptococci The utilization of acetate for acetylation by crude pigeon liver preparations and the system derived from *Neurospora crassa* is in accord with the finding in this laboratory that radioactive acetic acid is incorporated into the acetyl group of H.A The data discussed in a later section of this

review on mammalian skin suggest a similar situation obtained in the synthesis of H.A. and the sulfated mucopolysaccharide of rabbit skin

Before leaving the subject of the enzymatic reactions of glucosamine, mention should be made of the demonstration by Park (1952 a, b) of a uridine nucleotide which contains an amino sugar and the more recent isolation by Cabib et al. (1953) of a uridine diphosphate acetylglucosamine compound It is suggested that this compound may play some role in the metabolism of hexosamines although this has not so far been established. Importance of note in this connection is the recent demonstration of the role of the homologous uridine derivative, uridine diphosphate glucose, in the synthesis of sucrose (Leloir & Cardini, 1953 b).

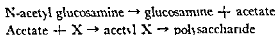
Biosynthesis of Mucopolysaccharides

On the basis of the known structure of the acid mucopolysaccharides it *is obvious that many possible pathways of biosynthesis may be postulated.* Final decision regarding pathways can only be obtained after isolation and study of the enzyme systems involved. Some data are, however, now available pertinent to these questions These results have been obtained to a large extent using carbon-14 tracer techniques in the study of the synthesis of H A. by a strain of Group A streptococcus (Dorfman et al, unpublished results)

A first approach was attempted utilizing carboxyl labeled N-acetyl glucosamine, since the demonstration of the incorporation of this compound would obviate the necessity of the study of the incorporation of glucosamine. When N-acetyl glucosamine, labeled in the carboxyl group, was added to a semisynthetic medium containing glucose, the H.A. produced by group A streptococci was found to be labeled in the acetyl group, thus suggesting that N-acetyl glucosamine could indeed be directly incorporated. Such a conclusion had previously been reached by Topper & Lipton (1953)

This conclusion, however, seemed open to question when the following facts were ascertained When labeled N-acetyl glucosamine was added to the medium acetate isolated from the growth medium was found to be highly radioactive. Furthermore, when carboxyl labeled acetic acid was incorporated into the medium, the acetyl group of the H.A. was found to be highly radioactive. These findings suggested that the apparent incor-

poration of N-acetyl glucosamine may actually be due to the following set of reactions:


N-acetyl glucosamine → glucosamine + acetate
Acetate + X → acetyl X → polysaccharide

X may be glucosamine or any other intermediate (such as a disaccharide or phosphorylated glucosamine intermediate). To test this hypothesis additional experiments were performed in which inactive acetic acid was added in addition to the carboxyl labeled N-acetyl glucosamine. It was reasoned that if N-acetyl glucosamine is directly incorporated into the polysaccharide, the presence of inactive acetate should not affect the degree of labeling, while if the mechanism proposed above is responsible for labeling, the presence of inactive acetate should result in a decrease of the radioactivity of the H A formed. The latter was found to be the case, thus making impossible the conclusion from this type of experiment that N-acetyl glucosamine is a direct precursor

Further experiments were performed to determine whether glucosamine is utilized for polysaccharide synthesis For this purpose C^{14}, N^{15} doubly labeled glucosamine was prepared by incorporating 1-C^{14}-glucose and $N^{15}H_4^+$ in a medium upon which was grown *Aspergillus niger* The doubly labeled glucosamine which was isolated from the chitin of the mycelium was utilized for the synthesis of H A by the streptococcus The ratio of C^{14}/N^{15} was found to be almost identical in the glucosamine placed in the medium and the glucosamine isolated from the H A, thus unequivocally demonstrating that glucosamine is a precusor of H.A

The origin of the glucuronic acid portion of the polysaccharide molecule is not clear. Incorporation of labeled glucuronolactone into the growth medium has so far not resulted in incorporation of radioactivity into the molecule

Metabolism of Mucopolysaccharides in the Mammal

It is obvious from the preceding discussion that of the meager information available regarding the metabolism of mucopolysaccharides, little has been derived from study of mammalian connective tissue This deficiency derives to a great extent from the difficulty of securing adequate material for such studies. A possible exception to this is cartilage.

The degradation of the acid mucopolysaccharides by the enzyme hyaluronidase will be considered in another chapter of this publication, and for the purpose of this section will not be considered as playing a role in the synthesis or degradation of H.A or C.S A. in connective tissue This assumption is in keeping with the fact that there has so far been no conclusive demonstration of the presence of hyaluronidase in mammalian tissues other than testes

There is as yet no certain definition of the cells responsible for the synthesis or degradation of the mucopolysaccharides in connective tissue. It seems reasonably certain that the cartilage C.S A (chondroitin A of Meyer & Rapport, 1951) is produced by chondroblasts. It has been widely assumed that H A is produced by fibroblasts although no definitive proof of this has been presented Asboe-Hansen (1950) has suggested instead that mast cells are responsible for the formation of H.A. That this cell plays a role in the formation of heparin was suggested some time ago by Jorpes (1947) and has been accepted by many because of the presence of metachromatic granules in these cells. It is beyond the scope of this paper to consider the problems of cellular origins in detail.

The availability of S^{35} presents a highly advantageous tool for the study of metabolism of mucopolysaccharides. This isotope has many advantages for such studies, among which can be listed the following: 1) it is relatively cheap and easily available, 2) its low energy β emission is relatively easy to count and presents a minimum of health hazard, 3) it can be readily obtained as sulfate and used as such without performing further synthesis, 4) incorporation as ester sulfate can be readily determined by hydrolysis as precipitation of $BaSO_4$, and 5) the localization of the ester sulfate can be achieved by radioautography.

These advantages are countered by certain disadvantages such as: 1) this method gives no information regarding the metabolism of H.A., 2) conclusions must be confined to changes of the sulfate ester group, since it is possible that these are not correlated with changes in other parts of the molecule, 3) there is as yet no clear definition of all of the ester sulfate compounds present. Thus, the existence of more than one type of C S A. may result in confusion regarding conclusions with respect to metabolism. The presence of heparin poses similar problems. However, despite these difficulties considerable useful information has recently accumulated as a result of studies utilizing radioactive sulfate. Studies utilizing radioactive sulfate are discussed in a separate chapter of this book.

In view of the limitations of the information that can be obtained by the use of S^{35}, a series of studies have been undertaken in the author's laboratory utilizing C^{14} either alone or in combination with S^{35}. Before studies of metabolic rates could be undertaken, it was necessary to devise methods for the isolation of the acid mucopolysaccharides free of other contaminating carbon compounds as well as their separation from each other. Schiller et al (1954) have devised methods for the preparation from rabbit skin of both H A. and a sulfated mucopolysaccharide of high analytical purity. By all available methods the H A. behaves like that isolated from other sources, but the sulfated fraction shows certain discrepancies in behavior from cartilage C S.A. These are a lower colour equivalent by the carbazole reaction and a different behavior toward testicular hyaluronidase The exact nature of the sulfated mucopolysaccharide has not as yet been elucidated

Using such methods it has been possible to study rates of incorporation of C^{14} following the administration of carboxyl labeled acetate and 1-C^{14}-labeled glucose In the first set of experiments C^{14} carboxyl labeled acetate was administered in divided doses for various intervals The rabbits were sacrificed at the end of the periods and the H.A and sulfate polysaccharides were isolated from the skin of the individual animals for radioactivity analyses The rate of incorporation of C^{14} into H.A was found to be approximately 3 times as rapid as that observed in the sulfated mucopolysaccharides In a single animal to whom 1-C^{14}-glucose was administered over a period of 5 days, the radioactivity of the isolated polysaccharides bore the same relationship

In another set of experiments approximately 0.75 mC of carboxyl-labeled acetate was administered in divided doses on one day and pairs of animals were sacrificed on the first, fourth, eighth and twelfth days after the beginning of injections The initial radioactivity (18 hours after the last administered dose) of the H.A was found to be considerably greater than that of the sulfated polysaccharides The rate of decrease of this activity was much greater for the H A Actual calculation of half-life time was not satisfactory because of the complexity of the kinetics involved over the period of the experiment Assuming first order kinetics, however, a half-life time of about 2 days for H A and a considerably longer period for the sulfated polysaccharides was indicated. When polysaccharides from duplicate animals for each time period were pooled and degraded, it was possible to demonstrate that almost all of the radioactivity appears in the acetyl group

This is in keeping with the previous demonstration of the utilization of acetate for the N-acetyl group in the synthesis of H.A. by Group A streptococcus, and the relative inefficiency of acetate as a precursor for glucose (which presumably acts as the precursor of the hexosamine and glucuronic acid portions of the polysaccharide molecule).

The finding of a rapid rate of turnover of H A. in the skin of adult rats is somewhat surprising in view of the usually assumed stability of connective tissue. It, however, makes more understandable the known rapidity of changes that apparently occur under certain physiological and pathological stimuli

The marked discrepancy of turnover rate in the H.A. and the sulfated polysaccharides deserves special comment. It is possible that the sulfated polysaccharide isolated from skin may represent a mixture of substances, thus the apparent turnover rates would be a function of more than one rate. It is apparent, however, that at least one sulfated substance is present which turns over much more slowly than H.A. The actual rates observed appear to be of the same order of magnitude as that observed by Bostrom & Gardell (1953) utilizing S^{35}.

Whether the rates of turnover measured with either acetate or sulfate represent total breakdown and resynthesis of the polysaccharide or merely exchange of the groups is not as yet clear.

Summary

The review of the literature which has been presented makes it obvious that knowledge regarding metabolism of mucopolysaccharides is as yet in a very fragmentary stage of development. It is equally apparent that with application of biochemical methods which have yielded rapid progress in other fields of biochemistry, a better understanding of the physiology and biochemistry of connective tissue should soon become available It is still too early to predict the usefulness of such information in bringing about an understanding of the rheumatic and hypersensitivity states, but it seems reasonable to assume that such information should improve our understanding of the physiological and pathological behaviour of connective tissue.

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ON THE SULPHATE EXCHANGE OF SULPHO-MUCOPOLYSACCHARIDES

AN ENZYMATIC REACTION IN MESENCHYMAL TISSUES

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Introduction

THE VARIOUS FORMS of collagenoses are manifested by involvement of mesenchymal tissues of different kinds, such as cartilage, connective tissue, tendons or vessel walls. These processes often cause severe pathological changes in the mesenchymal tissues; it may therefore be presumed that they also give rise, in some way or another, to metabolic disturbances in the chemical constituents of these tissues. Consequently, studies of the metabolism of these substances under normal and pathological conditions can be expected to contribute to our knowledge of the pathogenesis of the collagenoses

Modern biochemical research has shown—particularly with the use of isotope tracer methods—that most constituents of the body are in a dynamic state Different compounds are destroyed and re-synthesized at rates more or less characteristic of each compound

Important chemical constituents of the mesenchymal tissues are certain proteins, *e g*, collagen and elastin and different kinds of mucopolysaccharides, such as hyaluronic acid, chondroitin sulphuric acid, mucoitin sulphuric acid and heparin Our knowledge of the metabolism of these compounds is, however, fairly incomplete With regard to the metabolic activity of collagen, it was demonstrated by Neuberger et al (1951), by means of the administration of C^{14}-labelled glycine, that this activity is extremely low in the tendons of adult rats This indicates that, once deposited in the intracellular spaces, collagen probably becomes metabolically inert.

As far as the mucopolysaccharides are concerned, most studies on the metabolism of these compounds have been confined to problems dealing with the enzymatic exchange of the sulphate groups of sulpho-mucopolysaccharides. These studies have been made with the use of S^{35}-labelled sulphate as the tracer substance and have been reviewed recently (Bostrom, 1953, Boström & Jorpes, 1954); they will be discussed briefly in the following

Sulphate Fixation in Vivo

If a tracer dose of S^{35} as sulphate is given to an experimental animal, most of the radioactive sulphate is rapidly excreted (Dziewiatkowski, 1949), only a small fraction being retained in the tissues

After the pioneer work of Dziewiatkowski et al. (1949), who demonstrated a very high sulphate incorporation and a slow elimination of S^{35} in cartilage, bone and bone marrow, numerous investigations have been made to determine the exact localization in the tissues of the retained part of the radioactive sulphur

The autoradiographic method has proved to be eminently suitable for this purpose. By means of this technique, the radioactive sulphate fixed in a large number of normal organs and tissues has been visualized in the rat and the rabbit.

In the *skeleton,* the highest sulphate fixation was noted in different kinds of cartilage (Dziewiatkowski, 1951 a, Campbell & Persson, 1951, Bostrom et al, 1952) but a marked uptake was also found in bone tissue, with a maximum in the periosteal and endosteal layers and certain Haversian systems (Engfeldt et al., 1954).

The *skin* showed a low uptake of S^{35} in the cornified layer. In the corium, an even distribution of somewhat more S^{35} was observed, interrupted by the pictures of hair follicles and small vessels, which showed a still higher concentration (Bostrom et al, 1953). A remarkably high sulphate fixation occurred in the mast cells of the subcutis, in contrast to the extremely low fixation in other parts of this layer (Jorpes et al., 1953, Asboe-Hansen, 1953)

In the *respiratory tract,* the highest sulphate fixation was found in tracheal and bronchial cartilage and in the cartilage plates of the lung, whereas other structures showed a low or moderate uptake.

In all the sections of the *gastro-intestinal tract* investigated, the highest

degree of sulphate fixation was exhibited by the epithelial layer In other layers of the oesophagus, stomach and intestines and in the liver and pancreas, a diffuse low or moderate uptake was noted.

In the *cardiovascular system,* the highest S^{35} incorporation occurred in the aorta, in the walls of the arteries and in the heart valves. The heart muscle and the spleen showed only a moderate diffuse uptake (Odeblad & Bostrom, 1952 a).

Well-differentiated autoradiographs have also been obtained from the nervous system, the genito-urinary system, the eye, the ear and certain embryonic tissues, they indicate that the S^{35} incorporation varies markedly in different structures (Bostrom & Odeblad, 1953 a, b, Odeblad & Bostrom, 1952 b, Belanger, 1953)

With regard to the mode of sulphate fixation, it has been shown by isolation of labelled chondroitin sulphuric acid from the cartilage (Dziewiatkowski, 1951 b, Bostrom, 1952) and skin (Bostrom & Gardell, 1953) of rats given injections of S^{35}-labelled sulphate, that most of the radioactive sulphur in these structures is deposited as ester sulphate in chondroitin sulphuric acid Isolation from the same animals of other sulphur-containing compounds such as cystine, taurine and methionine showed, however, only a low or inappreciable uptake (Bostrom & Åqvist, 1953). The main part of the fixed radioactive sulphate demonstrated in other tissues by means of autoradiography occurs in those tissues which are known to contain sulpho-mucopolysaccharides Consequently, it may be suggested that, in these tissues as well, large amounts of radioactive sulphur occur in esterified mucopolysaccharides, *e.g* heparin in the mast cells, mucoitin sulphuric acid in the mucus of the gastro-intestinal tract and the chondroitin sulphuric acid in vessel walls and the heart valves, for example

It seems possible to make the following inference from the high uptake of sulphate in different tissues of full-grown adult animals In different kinds of sulpho-mucopolysaccharides, destruction and re-synthesis of these polysaccharides, or more probably an exchange of the sulphate groups of these compounds, takes place continuously The rate of these processes has been directly studied in rats; the biological half-life time of the sulphate group of chondroitin sulphuric acid in cartilage and skin was found to amount to 16 and 10 days, respectively (Bostrom, 1952, Bostrom & Gardell, 1953).

Various factors seem to influence the sulphate exchange of sulpho-

mucopolysaccharides *in vivo*. Thus Dziewiatkowski (1951 c) reported a higher sulphate fixation after treatment of rats with thyroxin, whereas cortisone was found to decrease the rate of this process (Layton, 1951, Bostrom & Odeblad, 1953 c). In addition, it has been demonstrated that the sulphate exchange of chondroitin sulphuric acid and the sulphate fixation in different tissues is considerably decreased in scorbutic animals (Reddi & Norstrom, 1954, Friberg & Ringertz, 1954). Finally, it was recently shown by Ellis et al (1953) that hypophysectomy of immature growing rats results in a markedly diminished sulphate uptake in costal cartilage as judged from the cartilage to plasma inorganic sulphate specific activity ratios. Moreover, the injection of growth hormone partially restores this ratio to normal

Sulphate Exchange in Vitro

Layton et al. (1950), using a tissue culture technique, found a highly differentiated sulphate fixation in several surviving tissues. Bostrom & Månsson (1953 a) elaborated an *in vitro* slicing technique for the study of the sulphate exchange of chondroitin sulphuric acid of cartilage. Using the latter technique, fresh costal cartilage from newly-killed calves was cut in thin slices, the slices were incubated in Krebs-Ringer-bicarbonate solution, containing S^{35}-labelled sodium sulphate, at a temperature of 37° C, in the presence of a gas mixture containing oxygen and carbon dioxide After a few hours the reaction was stopped by boiling the samples, and chondroitin sulphuric acid of a high degree of purity was prepared from each sample. The radioactivity of the samples was then measured With this technique, a high S^{35} incorporation in the sulphate group of chondroitin sulphuric acid was easily demonstrated No such uptake of S^{35} occurred in slices boiled before incorporation, or when labelled sodium sulphate was added to pure chondroitin sulphuric acid alone.

The aforementioned method, which had a high degree of reproducibility, permitted studies on the influence of different factors on the sulphate exchange of chondroitin sulphuric acid *in vitro* Thus, it was found that freezing and subsequent thawing or heating of the cartilage to 47° C completely destroyed its ability to incorporate S^{35}. An increase in the rate of the reaction resulted from an increase in the incubation temperature until heat inactivation occurred. It was also demonstrated that this reaction could be inhibited by different kinds of enzyme inhibitors,

of which the SH inhibitors were the most potent. This may indicate that the enzymatic exchange of the sulphate groups of chondroitin sulphuric acid is related to SH enzymes present in the cartilage

Of certain interest was the observation that cortisone also strongly inhibited the sulphate exchange of chondroitin sulphuric acid *in vitro* (Layton, 1951, Bostrom & Odeblad, 1953 c). A similar effect was obtained with the use of salicylic acid as the inhibitor. As recently reported (Bostrom & Månsson, 1954), salicylic acid in concentrations comparable to the salicylate plasma levels obtained in patients treated with large doses of salicylic acid according to Coburn (1943) significantly inhibited the *in vitro* S^{35} incorporation in chondroitin sulphuric acid. On the other hand, the therapeutically inert isomers of salicylic acid, *i e*, the meta- and para-hydroxybenzoic acid, failed to inhibit this reaction

Another recent observation was that the sulphate exchange of chondroitin sulphuric acid *in vitro* could not only be inhibited by different agents, but could also be stimulated in different ways Thus, the presence of glucose (0 1 per cent) in the suspending medium gave an S^{35} incorporation in the samples of chondroitin sulphuric acid of cartilage which was 15–20 per cent higher than that in samples incubated without the addition of glucose to the Krebs-Ringer-bicarbonate solution

Stimulation of an entirely different order of magnitude was, however, recorded when small amounts of a liver homogenate were added to the slices (Bostrom & Månsson, 1953 b) In this case, radioactivity exceeding that of the control samples by more than 100 per cent was noted. It was shown in additional experiments that the active principle is present in the liver extract It is thermostable and can be dialyzed through a cellophane bag

Comments

It may be concluded from the studies on the sulphur metabolism of sulpho-mucopolysaccharides in mesenchymal tissues briefly summarized in the present paper that these compounds, like most other body constituents, are metabolically active Moreover, the metabolism of these compounds seems to be influenced by factors known to be correlated to mesenchymal tissues, *e g*. C-avitaminosis, cortisone, salicylic acid and hypophysectomy.

It must, however, be pointed out that experiments on the sulphate exchange only do not permit any inferences with regard to the synthesis

of the whole molecule of the chondroitin sulphuric acid. Nothing is as yet known about the rate of renewal, nor about factors influencing the exchange of other parts of the molecule, the amino group, the acetyl group or the whole carbon skeleton On the other hand, it seems possible that the sulphate exchange studied reflects, in one way or another, the metabolic activity of the whole molecule.

In view of the close connection between the mucopolysaccharides and the collagen in the mesenchymal tissues, it may be suggested that further studies on the former substances will throw some light on the obscure group of diseases now called collagenoses

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SOME REMARKS ON THE SPREADING REACTION

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IF AN INFECTIOUS AGENT such as vaccine virus is injected intradermally, together with an extract of mammalian testicle (Duran-Reynals, 1928), or a filtrate of a culture of an invasive streptococcus (Duran-Reynals, 1933), a pronounced enhancement of the infection is observed This is due to the unique phenomenon of the spreading (Hoffman & Duran-Reynals, 1930, McClean, 1930). "The bleb flattens immediately and the inoculum spreads through the skin as fluid dropped in a blotter If forced spreading is tried, it is amazing to watch how rapidly the inoculum can be dispersed in a few seconds over a large skin surface under small pressure, while a much stronger pressure fails to dislodge the bleb formed by the control mixture". (Duran-Reynals, 1942)

Obviously, an obstacle, a barrier, has been overcome by the injected material We know today that the obstacle largely consists of the glue formed by the polymerized complexes of polysaccharides present in the ground substance of the mesenchyme, the active substance in the injected material is the best known and probably the most important of the spreading factors, namely, the enzyme hyaluronidase (Chain & Duthie, 1939, Meyer et al, 1937, Robertson et al., 1940) The degradation of the polysaccharides by the enzyme results in a *gel* being changed into a *sol*, and any substance injected with the enzyme will diffuse freely in this fluid medium. This depolymerization is manifested in the test tube by the prompt loss of viscosity of hyaluronic acid acted upon by the enzyme The degree of degradation of the substrate varies depending on the source of the enzyme but, correcting a past belief, the hydrolysis of hyaluronic acid does not proceed beyond the liberation of disaccharides, a further effect on the latter being due to other enzymes (Hahn, 1945).

It seems to be generally agreed that a pure hyaluronidase-hyaluronic acid interaction is operative only in the first phase of the spreading reaction, *and that other effects play important parts at later phases.* Among the latter, pressure, volume of the inoculum, and effects resulting in an increase in the permeability of blood capillaries with resulting spreading edema, were known for some time (Hechter, 1950). Other important actions more recently uncovered are that derivatives of hyaluronic acid (Duran-Reynals, 1952), or products from its partial depolymerization (Seifter & Baeder, 1954), exert a strong spreading effect. In the latter case, under certain conditions, the spreading is equivalent to that of 2,000 TRU of the enzyme, curiously, if the hydrolysis of hyaluronic acid is allowed to go beyond a certain limit, spreading is no longer obtained

Spreading tests in the dead animal permit elimination of several of the effects superimposed to the enzyme-substrate reaction (Hechter, 1950). Based on that fact a biological method for titration of hyaluronidase has been recently proposed The test consists of the intradermal injection of enzyme dilutions in freshly killed guinea pigs and measuring the spreads shortly after: good or close agreement was found between this and the *in vitro* methods currently used (Humphrey & Jacques, 1953). It would be, we believe, highly desirable that accurate biological methods, combined or not with *in vitro* techniques, were adopted to titrate the enzyme. For, the complexity of all the effects leading to pharmacological action, evolved by the enzyme injection, can only be fully measured by the spreading reaction in a living tissue and still much better in a living animal.

Other substances, chemically defined or not, are known which give the spreading reaction. Let us quote, among these spreading factors, those present in the mammary glands (Elliot & Turner, 1953), thyroid (Spinelli, 1932, Levine, 1949), azoproteins (Claude, 1935); some mercurial diuretics (Edlund & Linderholm, 1949), ascorbic acid (Robertson et al., 1941).

In a few cases, e g. in the case of azoproteins and ascorbic acid, the spreading effect can be explained because a depolymerizing effect on preparations of hyaluronic acid, and other mucopolysaccharides, has been shown The dynamics of hyaluronidase action is largely the cause of the almost exclusive attention of workers to the polysaccharide components of the ground substance Yet, protein is an important component of it (Day, 1952). Simple observations such as the rapid spreading in the ground substance of fluids such as alcohol, ether, chloroform, etc., contrasting with

the behavior of water or saline under the same circumstances, are extremely revealing in this respect (Day, 1949). It seems likely that knowledge on the mechanism of action of spreading agents other than hyaluronidase would throw light on important phenomena in the ground substance

The spreading reaction is performed in the skin for the simple reason that there is present in the dermis a relatively thick continuous layer of ground substance and also because the results of the spreading can be so easily appraised. Yet, spreading reactions with hyaluronidase or even other spreading factors can be carried out in other organs such as in stomach and intestine, mammary gland, pancreas, etc. (Favilli, 1935, Duran-Reynals, 1942) Here again, contrasting with the extensive information we have on dermal spreading, what is known of spreading in other tissues is meager indeed

Yet, it is logical to expect that important information would be obtained if the spreading reaction was added to current electron microscopic and histochemical methods—including enzymatic treatment of tissue slices —for the sudy of the intercellular materials of normal and pathological tissues Some work in this direction has already been done, e.g the studies on the sex skin of monkeys by Duran-Reynals, Bunting & van Wagenen (1950), which coordinated results from the spreading reaction in the sex skin—and ordinary skin, too—with histochemical data and endocrine effects, those of Woodin (1950) to the effect that the spreading reaction fails to take place in the rabbit and ox cornea, a conclusion to be related to the nature of the polysaccharides in the organ and calling for a revision of previous statements on that point, and those of Pirie (1949) and von Sallman (1949) on the results of injection of hyaluronidase and dyes in the posterior chamber of the eye: diffusion is slow, but hyaluronic acid remains disaggregated for at least a month, an event strongly contrasting with the rapidity—3 days— of the reconstruction of the dermal barrier after the spreading reaction in man or animals (Romanoff, 1938, Hechter, 1948, Bywaters et al., 1951), and suggesting differences in the functioning of both sorts of ground substance. However, it seems clear that much more should be done along these lines For instance, in view of the newer knowledge on the content of polysaccharides in the matrices of the blood vessels, and on the effect of hyaluronidase on the deposition of cholesterol in their walls (Rinehart & Abul-Haj, 1951, Cali, 1952, Seifter et al, 1953), it could be very fruitful to study the spreading reaction in different segments

of the normal or pathological vascular system; the long debated problem of the effect of hyaluronidase on the permeability of the blood capillaries (see Duran-Reynals, 1942) would be a part of the study. Although in a more remote sphere, one could envisage comparable studies in the peripheral and central nervous system.

The same enzyme-substrate effect, which is the core of the spreading reaction, is also back of phenomena apparently so dissimilar as the following:

(a) The disaggregation of the granulosa cells surrounding the ovum as a preliminary step to fertilization (Fekete & Duran-Reynals, 1943, Mc Clean & Rowlands, 1942, see Duran-Reynals, 1942).

(b) The enhanced absorption of materials injected therapeutically under the skin or other tissues as in a clysis or in local anesthesia (Sanella, 1940, Kirby et al, 1950, Burket & Gyorgy, 1950, see Duran-Reynals, 1942, and Hechter, 1950)

(c) The rapid absorption of solutions injected into the joint cavities (Seifter et al., 1949).

(d) The quick diffusion of solutions through the urinary bladder by abolishing its semipermeable character (Seifter et al, 1949).

(e) The accelerated passage of saline solution through a muscular fasciae mounted as a filter *in vitro* (Day, 1952).

(f) The invasion of tissues by streptococcus as in a cellulitis or by *Cl. welchii* as in gas gangrene (see Duran-Reynals, 1942).

(g) The controlling of keloid recurrence (Cornbleet, 1954).

Still other examples could be given; they all serve to emphasize the universality of spreading reaction.

A number of observations recently accumulated show that when one of the many effects exerted by hyaluronidase such as above listed is antagonized by a given factor acting systemically, such as hormones, physical stress, infection, other phenomena are likewise antagonized. The examples that follow will illustrate the point:

(a) The spreading reaction in mice is suppressed, under certain conditions, by adrenal cortical hormones (Opsahl, White & Duran-Reynals, 1948, Opsahl, 1949). These hormones also suppress the permeability of the urinary bladder (Seifter, Baeder & Dervinis, 1949) and synovial mem-

brane (*Seifter, Baeder & Bogany, 1949*) *countering the effect of hyaluronidase* in these structures;

(b) The permeability of the dermal ground substance is rapidly lowered in infection induced by streptococcus or staphylococcus (Duran-Reynals & Estrada, 1940). The same infectious processes in the skin, or injury inflicted on a joint, result in a delayed absorption of materials introduced into a joint not subjected to injury (Edlund, 1949); and

(c) Estrone induces the typical changes in the epithelial and connective tissues of the reproductive organs. The hormone also causes the development of large amounts of ground substance in the sex skin of monkeys (Duran-Reynals, Bunting & van Wagenen, 1950), and it is of interest to mention here that, to judge by the published illustrations, the "tissue fluid" known to accumulate in the uterus during estrogen treatment since the pioneer work on the "follicular hormone" (see Allen, Hisaw & *Gardner, 1939*) *is in all probability newly formed ground substance* On the other hand, the hormone suppresses the spreading reaction and the dermal infection by vaccine virus (see Sprunt, 1950), and it also alters the permeability of the urinary bladder treated with hyaluronidase (Seifter, Baeder & Dervinis, 1949) If, instead of estrone alone, all the hormones contributing to the sex cycle and pregnancy are considered, other and more complex phenomena concerned with spreading, absorption from joints, granuloma formation (Meyer, Stucki & Aulsebrook, 1953) absorption from the skin (Ploman, 1953) and others could be found paralleling the sex changes

From these, as from still other examples that could be given, an important concept seems to be emerging, namely, that of the *unity of action* of the mesenchyme which can be expressed as follows· ***when an effect is exerted on a given sector of the ground substance it is likely that the same effect will also be observed, although manifested in a different way, in another sector of the same ground substance.***

This situation, expressed by the above law, holds true despite the undeniable fact that there is, at least grossly, a *selectivity of tissues* to hormonal effects, a subject closely connected with that of the *target cells* to endocrine actions in given animal species Further discussion of these points would involve the topic on the secretion of components of the ground substance and mechanisms of hormone effects, points which are analyzed in another chapter of this monograph. One point, however, must be emphasized, namely, that *all the hormonal effects so far studied are accompanied*

by pronounced quantitative or qualitative changes in the ground substance which seem to culminate in certain tissues of election. In other words, the *reactions of the mesenchyme are both systemic and selective.*

The spreading reaction is currently carried out by injecting intradermally hyaloronidase mixed with an indicator dye, this being controlled by an injection of the indicator mixed with saline; in most, if not in all cases, the latter injections disclose—although on a much smaller scale—the same changes of permeability of the ground substance as revealed by the hyaluronidase mixtures Representative examples are the results obtained after spinal section, exposure to cold (Homburger, 1943; Linderholm, 1951, *Birke*, 1953), injection of several hormones, and in tubercular, streptococcal and staphylococcal infection (see Duran-Reynals, 1942, Asboe-Hansen, 1952, Ducommun, Timiras & Dordoni, 1951; *and many others) In other cases events such as the pressure needed to* force the inoculum through the skin (Winter & Flataker, 1952) give values parallel to those obtained in the spreading reaction.

If the dermal ground substance is very permeable, as in very young rabbits (Duran-Reynals, 1942) or in guinea pigs with vitamin C deficiency (Persson, 1953), the addition of hyaluronidase to the injected dye adds little or nothing to the already large spreadings obtained by injecting the dye alone. In other words, *there are states in which the ground substance behaves as it does after hyaluronidase, or other spreading factors, have been injected into it.*

This statement, although not original, is timely. For, often one reads of "antihyaluronidase effects" ascribed to hormones, drugs or other factors which suppress the spreading reaction. Yet, in most cases at least, the essence of the phenomenon is simply an increase of the barrier value of the ground substance, this barrier being effective, although in different degree, against materials containing or not hyaluronidase. One must recognize, however, that in all probability there are cases in which the restriction of the spreading reaction is the result of an effect against hyaluronidase, as in the case of rabbits immunized against rattlesnake venom (Duran-Reynals, 1939; Tarabini-Castellani, 1938), or against preparations of *Cl. welchii* (McClean, 1936). In these cases the phenomenon is the result of a direct or indirect antibody effect against the enzyme; one can then imagine that among the reported cases there are some in which the suppression of the spreading is also due to effects of other nature on the enzyme.

The above paragraphs have dealt with the influence of systemic factors on the ground substance acted upon by hyaluronidase (or other factors) during the spreading reaction. In these last remarks we shall comment on what appears to be the opposite situation, namely, the influence that products liberated during the spreading reaction may have on systemic physiological phenomena A clinical observation is responsible for this newer aspect of the physiopathology of the mesenchyme The observation is that treatment of long duration with hyaluronidase prevents the recurrence of kidney stones of humans (Butt & Hauser, 1952) Since earlier studies (Butt and others, 1954) have shown that there was an inverse relation between the incidence of urolithiasis and the concentration in the urine of protective colloids in different sexes, races, and in changing physiological and nutritional conditions, and since the injection of hyaluronidase is thought to be promptly followed by an increase of these protective colloids, it has been concluded that the enzyme acts by liberating these colloids from the ground substance during the spreading reaction Although, obviously, much more work is needed to reach final conclusions on the nature of the substances involved in the phenomenon and on the nature of the protective effect, one fact seems to be established *the induction of an effect at distance as a consequence of the spreading reaction.* This effect is manifested by a therapeutic action on lithiasis, but it would seem logical to think that this particular renal action is only a manifestation of a far more generalized phenomenon which could, perhaps, be qualified as the *second and systemic phase of the spreading reaction which would follow its first, local phase.* It would also seem logical to think that hyaluronidase is not the only substance capable of inducing these systemic effects by acting on the ground substance, other spreading factors, some hormones, and still other agents, which are known to act on the ground substance of the mesenchyme can, through this local effect, induce important systemic actions Knowledge of these actions may turn out to be of great interest in both physiology and pathology

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INHIBITION OF HYALURONIDASE

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Introduction

THE PRESENCE OF LESIONS in the connective tissues has focused attention on the possibility that biochemical changes in these tissues may afford a clue to the understanding of the etiology and pathogenesis of rheumatic diseases and "hypersensitivity" states. Unfortunately, knowledge regarding the biochemistry and physiology of connective tissue is so limited that a systematic approach to this problem has so far not been possible.

The striking *in vivo* and *in vitro* effects of the enzyme, hyaluronidase, has resulted in many attempts to implicate this enzyme in the pathogenesis of rheumatic disease Impetus to such explanations has been derived from the existence in human blood serum of a substance which inhibits hyaluronidase and from the fact that various physiologically and pharmacologically active substances inhibit hyaluronidase It is the purpose of this paper to attempt to critically review some of the many recent studies which have concerned themselves with the problem of the inhibition of hyaluronidase. Before proceeding to a detailed discussion of these various inhibitors, it would seem necessary to delineate the subject matter and review certain general considerations pertinent to such studies.

In discussing the inhibition of hyaluronidase, it is necessary to distinguish between *in vivo* and *in vitro* effects. Although this review will be concerned almost entirely with *in vitro* studies, certain comments regarding *in vivo* studies would seem pertinent. The now well established "spreading effect" of hyaluronidase leaves no doubt that this enzyme brings about an increased rate of "spread" of many foreign substances in the connective

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tissues It should be pointed out, however, that although hyaluronidase is a most effective spreading factor, other substances have also been demonstrated to have such effect (Duran-Reynals, 1942). In addition to the inhibition of spreading phenomenon by various substances, it has now been well demonstrated that many physiological factors are involved in determining the rate of spread, so that apparent inhibition or stimulation of spreading may result from factors other than direct effect on hyaluronidase. Thus, the variable effects of hormones and drugs on the spreading reaction may be completely unrelated to the effects of the enzyme, but may be rather concerned with changes in the state of the ground substance such as might be brought about by differences in hydration, concentration of polysaccharides, type of polysaccharides, nature of proteins, etc No attempt then will be made in this discussion to consider the many factors affecting the spreading reaction

Before leaving the question of the *in vivo* effects of hyaluronidase certain comments regarding the physiological or pathological role of the enzyme should be made Although the biological effects of hyaluronidase in certain experimental situations are very striking there is no evidence that hyaluronidase plays any significant role in the normal metabolism of the polysaccharides of connective tissue Hyaluronidase has not been conclusively demonstrated to occur in the mammal in locations other than testicular tissue It is obvious that there must be other enzyme systems which are concerned with synthesis and breakdown of mucopolysaccharides, but their exact nature is as yet unknown These statements should illustrate the dangers of drawing conclusions regarding the mechanism of action of drugs in rheumatic diseases on the basis of their inhibition or lack of inhibition of hyaluronidase Similar objections apply to teleological explanations regarding the role of the hyaluronidase inhibitors of blood

The interpretation of the possible importance of various hyaluronidase inhibitors depends to some extent upon the validity of the *in vitro* demonstration of hyaluronidase. In view of the complexity of the *in vitro* assay methods, the first part of this review will be concerned with the assay of the enzyme and the various factors which may affect the activity of the enzyme.

A large, and often contradictory, literature has grown up on the subject of factors influencing hyaluronidase activity In order to establish a basis for generalization it is important to classify and systematize all pertinent experimental findings. However, it is regrettably necessary to omit from discussion here many papers peripheral to the subject, and others in which

the data are inadequate to allow critical evaluation. The literature on hyaluronidase has been reviewed recently by Meyer & Rapport (1952) and by Gibian (1951), who includes a complete listing of publications in this field.

Hyaluronic acid, the enzyme substrate, is a high polymer (probably *straight chain) composed of equimolar quantities of acetylglucosamine and* glucuronic acid residues joined by β-glycosidic links. Hyaluronidases may be defined (after Meyer & Rapport, 1952) as enzymes which depolymerize or hydrolyze hyaluronic acid, presumably by splitting glucosaminidic bonds. Glucuronidases are distinctly different enzymes which open glucuronidic bonds Hyaluronidases have been obtained from many sources including mammalian testes, various species of microorganisms, and secreta of lower animals. The great majority of reports, however, have dealt with the enzyme obtained from mammalian testes. Although this enzyme, in contrast to that obtained from bacterial sources, also acts upon chondroitin sulfuric acid from hyaline cartilage, hyaluronic acid has been virtually exclusively used as a substrate. It is therefore with the hydrolysis of hyaluronic acid by testicular hyaluronidase that we are mainly concerned.

Enzyme Assay

Although hyaluronidases have been assayed in large variety of ways only two procedures, viz. viscosity reduction (V.R.) and turbidity reduction (T R.) yield values that correlate well with each other. Not only standard procedures, but also similar preparations of enzyme and substrate are essential if results among different laboratories are to be compared. Testicular hyaluronidase may exist in different active states (Mathews & Dorfman, 1954) and appears to be associated with "carrier" protein (Malmgren, 1953). The influence of salts and substrate impurities upon the assay is emphasized by Meyer & Rapport (1952).

Some additional factors should also be considered. Since there is no general agreement by workers in this field on a standard hyaluronidase unit, the effect of an additive on hyaluronidase can be measured only by a relative, or percentage change in enzyme activity. The use of a "standard" enzyme preparation would facilitate the comparison of data obtained in different laboratories. Since widely different environmental conditions are used in determining the effect of an additive upon hyaluronidase, divergent

results may be obtained. Serious consideration should be given to the employment of a "standard" inhibitor, possibly in the manner in which Hahn & Fekete (1953) have used resorcinol

"Activators" of Hyaluronidase

Rogers (1948) noted that extreme dilution of a concentrated preparation of *Cl welchii* hyaluronidase led to a rapid loss of enzyme activity Dilution with either gelatin or peptone was partially effective in preventing inactivation while gum arabic at 0 5 per cent apparently gave complete protection. Mathews, Roseman & Dorfman (1951) found it necessary to dilute the very highest purity testicular hyaluronidase with bovine serum albumin to avoid loss of activity during assay Less pure preparations diluted to give solutions of the same activity per ml. did not require the added protein Subsequently it was noted (Alburn & Whitley, 1954) that other enzyme preparations of relatively low purity showed considerable apparent activation by hydrolysed gelatin

Meyer & Rapport (1951) observed that addition of pyrophosphate and especially of cysteine gave enzyme activities 30 to 60 per cent higher than that obtained without these additions This activation depended mostly upon the substrates Since testicular enzyme was known to be inactivated by Fe^{+++} and Cu^{++}, impurities frequently present in trace amounts in hyaluronic acid preparations, it appears likely that the "activation" was in reality a release of inhibition of the enzyme It is possible that a similar explanation accounts for the enzyme activating effects ascribed to protamine (Panthtschko & Kaiser, 1951), to nitrogen mustard (Costa, 1950) and to Ca^{++}, as well as to gelatin and albumin Alternatively, as suggested by the results of Malmgren (1953), it may be that hyaluronidase is a dissociable protein which requires a "carrier" for maximal activity Recent evidence of Alburn & Whitley (1954) and Diczfalusy et al. (1953), however, demonstrates clearly that protamine acts by a mechanism involving release of inhibition

Chemical Inhibitors

It is highly desirable to elucidate the mechanism of action of each additive which influences the rate of depolymerization of hyaluronic acid by hyaluronidase Since the kinetics of action of the enzyme are rather

complex, it has usually been difficult to satisfactorily approach this objective Nevertheless, it appears feasible to draw valuable inferences as to the probable mechanism of action of additives based upon some generalizations to be presented below.

For purposes of classification, it is necessary to draw a rather arbitrary distinction between inhibiting and non-inhibiting substances. It is assumed that substances which require a minimum concentration of 0.1 per cent (or 0.01 *M*) in order to produce appreciable inhibition (20 per cent reduction of enzyme activity) are very probably agents which are not specific, i e. they combine with, or otherwise alter, large groups of proteins besides hyaluronidase For example, the requirement of high concentrations of sodium salicylate to demonstrate inhibition casts doubt on the specificity of action of this substance (Dorfman et al, 1947). Furthermore, although some borderline cases occur, most of the substances fall clearly into one or the other category It is necessary, however, to omit from consideration data on inhibition which either cannot be classified by the above procedure, or is deemed experimentally uncertain for reasons discussed earlier.

High Molecular Weight Compounds

Alburn & Whitley (1954) have recently obtained kinetic data which shows that hyaluronidase follows the Michaelis-Menten equation in forming an enzyme-substrate complex and that heparin inhibition is competitive. It had been earlier proposed (McClean, 1942) that heparin acted as a competitive inhibitor by virtue of its chemical structural similarity to the natural substrates hyaluronic acid and chondroitin sulfuric acid A similar explanation would account for the inhibitions observed for sulfated hyaluronic acid (Pantlitschko & Kaiser, 1951), acetylated and nitrated hyaluronic acid (Hadidian & Pirie, 1948; Follett, 1948), and various other derivatives of hyaluronic acid which, like heparin, are strongly bound to, but not depolymerized by hyaluronidase.

Also to be included in the above group of high molecular weight compounds, structurally similar to the enzyme substrates, are probably cellulose trisulfuric acid and chitin disulfuric acid (Astrup & Alkjaersig, 1950), sulfated xylan and sulfated carboxycellulose (Pantlitschko & Kaiser, 1951).

Guerra (1946) claimed that the administration of salicylates resulted in the decrease of the spreading reaction produced by hyaluronidase and concluded that this effect may be pertinent to the mechanism by which sali-

cylates favorably affect rheumatic diseases. It was subsequently demonstrated (Dorfman et al., 1947) that inhibition of hyaluronidase is brought about only by concentrations of salicylates sufficient to act as a non-specific protein denaturant Meyer et al. (1948) demonstrated that certain preparations of gentisic acid, a metabolite of salicylates, inhibited hyaluronidase but later work by Roseman & Dorfman (1952) established the fact that the inhibition by gentisic acid was due to impurities Treatment of gentisic acid with alkali in the presence of oxygen results in the formation of a polymer which is an active inhibitor. Similar results have been reported by Forrest et al. (1952).

Along similar lines Hahn (1952), Hahn & Fekete (1953), Hahn & Frank (1953) found polycondensed hydroxybenzoic acid derivatives to be potent inhibitors Other active polymers include alkali treated or oxidized flavanoids (Rodney et al., 1950), polymers prepared by condensation of formaldehyde and sulfonated phenols (Rogers & Spensley, 1954) polymeric phosphates of phloretin and polyphenols (Diczfalusy et al., 1953), and polymeric phosphates of various aromatic hydroxy and amino compounds (Fernö et al, 1953)

It is of interest that sulfated starch and sulfated glycogen do not inhibit hyaluronidase (Pantlitschko & Kaiser, 1951). Since these molecules are highly branched (for starch, this refers to the amylopectin fraction) it would appear that to be an effective inhibitor an anionic polyelectrolyte should also have a linear chain structure Sodium hexametaphosphate, a linear inorganic polyelectrolyte (Strauss et al, 1953), inhibits hyaluronidase from *vibrio cholerae* (Burnett, 1948).

Although anionic polyelectrolytes appear to be specific inhibitors of hyaluronidase, in the sense that their action is competitive with the substrate, these substances may also combine with proteins (possibly including hyaluronidase) in a non-specific manner The presence of multiple cationic groups in a protein allows for many points of strong electrostatic interaction with an anionic polyelectrolyte Heparin and suramin have been found to inactivate many enzymes other than hyaluronidase at pH's sufficiently low to charge basic protein groups An excellent discussion of the role of these substances as enzyme inhibitors is given by Myrbäck & Persson (1953). These authors made a detailed study of the inhibition of β-amylase by heparin The inactivation was strongly pH dependent, no inhibition being observed above pH 5 0. Below pH 5 0 an otherwise large inhibition could be prevented by an increase in buffer or sodium chloride concentration.

There was evidence for an irreversible inactivation which slowly increased with time. Synthetic anionic polyelectrolytes, which are strong hyaluronidase inhibitors, were found by Diczfalusy et al (1953) to also inhibit other enzymes, but not in a competitive manner. It would appear that anionic polyelectrolytes may interact with proteins in many ways.

Low Molecular Weight Organic Compounds

Many low molecular weight organic compounds have been investigated for inhibitory action against hyaluronidase. Some 70 of these substances with little or no potency are listed by Hahn & Fekete (1953). However, there remains a number of chemically unrelated substances which show a remarkable effectiveness at low concentrations. The following discussion is presented as an attempt to find a unifying principle to account for this behavior

Molecules of chondroitin sulfuric acid carry a large negative charge and possess an isotropically coiled configuration (Mathews & Dorfman, 1953; Mathews, 1953 a). Sulfated hydrocarbons with sufficiently large hydrophobic groups form in solution large molecular aggregates which are similar to chondroitin sulfuric acid molecules in size, in gross shape, and in high negative charge. Thus, one might expect a high affinity of hyaluronidase for such anionic micelles due to a similarity of the micelles to the enzyme substrate. A structural similarity of anionic detergent micelles and acid polysaccharides was also proposed by Levine & Schubert (1952) to explain the closely parallel behavior of these substances toward metachromatic dyes.

Experimental evidence in support of the above view was obtained by Mathews (1953 b) in an investigation of the effect on testicular hyaluronidase of an homologous series of sulfated aliphatic alcohols. The concentrations at which the detergents produced equal rates of enzyme inactivation correlated closely with the concentration at which the detergents formed detectible micellar aggregates (critical micelle concentration). It was suggested, therefore, that the micelle-forming properties of these compounds are of paramount importance in determining their affinity for the enzyme

The detergents act directly upon the enzyme by producing a time-dependent irreversible inactivation, observed upon incubation of enzyme and detergent This contrasts sharply with the behavior of the high mole-

cular weight polyanions which act competitively (Alburn et al., 1954). Incubation of enzyme with acetylated or nitrated hyaluronic acid derivatives (Hadidian & Pirie, 1948) or with condensation products of hydroxybenzoic acids (Hahn & Fekete, 1953) show very little or no increase of inhibiting effect with time. Unfortunately, direct effects upon the enzyme have not been satisfactorily investigated for other inhibitors.

Calesnick & Beutner (1949) investigated a series of substituted phenols for inhibitory action. They reported that the activity of these compounds is low unless appreciable hydrocarbon side chains are present in the molecule. For instance, hexylresorcinol is some 50 times as potent as resorcinol. However, the presence of a strongly hydrophilic group and a long hydrocarbon chain (hydrophobic) in one molecule is a sufficient condition to allow formation of micellar aggregates. Upon further investigation, it was possible to demonstrate that hexylresorcinol did indeed form such aggregates (Mathews, 1953 b) and that the action of hexylresorcinol was directly upon the enzyme with production of a time-dependent irreversible inactivation.

The compound, d-α-tocopheryl phosphate also possesses a structure characteristic of associative colloids. It resembles the detergents in producing a time-dependent enzyme inactivation but differs from them in that the inactivation can be reversed by dilution (Mathews, 1953 b). It is probable, however, that the action of d-α-tocopheryl phosphate likewise depends upon relatively non-specific micellar properties. Miller & Dessert (1949) had earlier noted the inhibitory action of various sulfated detergents and of tocopheryl phosphates. They had reported that a given inhibitor (sulfated detergent or tocopheryl phosphate, but not heparin) was more effective when mixed with enzyme before addition of substrate than when mixed with substrate before addition of enzyme. They had failed, however, to recognize that this inactivation of enzyme by inhibitor was a continuing action with time.

Aurin tricarboxylic acid is a small molecule with a remarkable effectiveness as an inhibitor of hyaluronidase at concentrations as low as $5 \times 10^{-8} M$ (Mathews, 1953 b). It's action is like that of heparin. Significant, however, is the fact that aurin tricarboxylic acid is highly associated in solution, as indicated by its failure to dialyze through a membrane permeable to small ions. Comparable behavior is shown by many dyes known to be associated in solution (Alexander & Johnson, 1949). It may be assumed that the effective unit for inhibition is a large colloidal aggregate of high negative charge. A similar assumption of molecular association would account for

the effectiveness of suramin and phosphorylated or sulfated hesperidin derivatives (Beiler & Martin, 1947, 1948), products of phosphorylation of hesperidin, rutin, quercitin and hydroxylated chalcones (Preston et al, 1953) and of various compounds containing both hydroxyl and carboxyl groups (Hahn & Fekete, 1953).

Wattenberg & Glick (1949) noted the inhibition of hyaluronidase by bile salts and sulfated sterols Bile salts are known to form anionic micelles (Ekwall, Lundstrom & Setala, 1951) and the sulfated sterols, on chemical structural grounds, would be expected to yield micelles also. These authors also reported that hemin, protoporphyrin and coproporphyrin were effective inhibitors Whether the reported inhibition of hyaluronidase (Dirscherl & Kruskemper, 1952) by cortisone and desoxycorticosterone acetate is related to associative properties of these substances is difficult to say in the absence of information on the mechanism of action.

A large number of enzymes including urease, invertase, peroxidase and amylases were reported by Hockenhull (1948) and by Wills (1953) to be inhibited by sulfated hydrocarbons and sulfated fatty alcohols provided the pH is sufficiently low (apparently below the isoelectric point of the enzyme). Myrback & Persson (1952) reported that the inactivation of β-amylase by sodium dodecyl sulfate increased with time of incubation and was apparently irreversible It is possible that the anionic detergents act in a similar manner upon many different proteins. Hyaluronidase differs from the above mentioned enzymes principally in that inactivation proceeds well above (i e. at pH 7.0) the isoelectric point of 5.7. The high affinity of hyaluronidase for inhibitory anions when the net charge on the enzyme molecules is negative may well be related to the presence of a protein structure suited to specific combination with polyanionic substrate molecules.

Metals

Fe^{+++}, Cu^{++}, Fe^{++} and Zn^{++} salts, in order of decreasing effectiveness at 5×10^{-4} *M*, are reported (Meyer & Rapport, 1951) to strongly inhibit testicular hyaluronidase. Fe^{+++} and Cu^{++} are active at concentrations as low as 10^{-5} *M* The group or groups in the enzyme binding the metal are not known At a concentration of 5×10^{-4} *M*, Cd^{++}, Ni^{++}, Co^{++}, Mn^{++}, Pb^{++}, Al^{+++} and Hg^{++} are inactive No effect on the enzyme was found for Ca^{++}, Mg^{++}, Ni^{++}, Co^{++}, $[Co(NH_3)_5Cl]^{++}$ and

$[Co(NH_3)_5H_2O]^{+++}$ at concentrations up to 5×10^{-3} *M* (Mathews et al., 1952). Hale (1944) found no effect of 2×10^{-4} *M* $CuSO_4$ on hyaluronidase from *Cl. welchii*

Non-Specific Inhibitor

This discussion is concerned with characteristics of those substances found in animal sera, irrespective of known previous exposure of the animal to the enzyme, which inhibit hyaluronidase *in vitro*. Although this substance (or substances) has usually been denoted as a physiological inhibitor, there is no evidence regarding its *in vivo* function Almost all of the studies to be discussed below have been carried out utilizing bovine testicular hyaluronidase

That the blood of a number of mammalian species brings about the inactivation of hyaluronidase has been suspected since Duran-Reynals (1933) demonstrated the disappearance of "spreading factor" from blood after intravenous injection Hobby et al (1941) found evidence of the inhibition by normal human and rabbit sera of hyaluronidase prepared from pneumococci, streptococci and *Cl welchii* They ascribed this effect to salt formation between serum albumin and hyaluronic acid, suggesting that the inhibition was primarily due to an effect on the substrate rather than on the enzyme McClean (1942) reported that hyaluronidases prepared from bull, rabbit and mouse testes were inhibited by guinea pig, rabbit, sheep, horse, mouse and human sera. Heparin, chondroitin sulfuric acid and gastric mucin were also found to be inhibitory, but the blood inhibitor was thought to differ from these on the basis of chemical properties.

Relatively little attention was paid to this phenomenon until a revival of interest occurred as a result of the publication of a series of papers by Haas (1946) who ascribed considerable physiological significance to the hyaluronidase inhibitor (antiinvasin I) and other postulated proinvasins and antiinvasins Haas' data and conclusions will be discussed in the appropriate sections below

Assay Procedures

The serum hyaluronidase inhibitor has been assayed by a variety of methods; most commonly used have been the V.R., T.R , methods, and less frequently the mucin clot prevention (M C.P) method The former

two methods give satisfactory results providing due regard is given to the various factors which affect the *in vitro* action of hyaluronidase The M C.P. method does not permit sufficient precision to demonstrate differences in inhibitor levels of the magnitude which have been demonstrated by other methods The lack of standardization of hyaluronidase activity or methods has precluded standardization of serum inhibitor studies Thus, interpretation of results in the literature depends upon internal consistency of the results of any one laboratory.

Some confusion in interpretation of the older literature was introduced by the method of calculation introduced by Haas (1946) and used in the early studies of Glick (1950). The theoretical objections to this formulation have been discussed by Dorfman et al. (1948) who suggested that activity of inhibitor be expressed in terms of the amount of enzyme inhibited under rigorously standardized conditions. Other authors (Glick, 1950; Adner, 1948) have preferred to express inhibitor activity as percentage of enzyme inhibited Both methods of calculation can be used with both the T.R. and V.R. methods It should be also noted that many of the published studies have not taken into account the necessity of magnesium for the action of the inhibitor (see discussion below) and have therefore not determined maximum activity of the inhibitor.

PURIFICATION

Early attempts to fractionate the inhibitory activity of human blood (Goldberg & Haas, 1947) led to the conclusion that the inhibitor could be separated into two components by alcohol fractionation However, Baumberger & Fried (1948), and Adner (1948) found that the loss of activity resulting from addition of oxalate and citrate could be restored by the addition of magnesium ion Freeman et al (1949) showed that the apparent separation into two fractions by alcohol fractionation is due to separation of magnesium, and almost complete activity could be recovered in *one fraction if magnesium ion were added to the assay mixture.* The effect of metal ions was studied in somewhat greater detail by Mathews et al. (1952) who showed that Co^{++} ion could replace Mg^{++} at even lower concentrations. The complex ion $[Co(NH_3)_5Cl]^{++}$ was also found to be active, while Zn^{++}, Ni^{++} and Mn^{++} prevent the action of the inhibitor but Ba^{++}, Sr^{++} and Ca^{++} were without effect.

Wattenberg & Glick (1952) were able to obtain preparations 130 times

purified with respect to the original plasma. Electrophoresis at pH 8 6 in veronal buffer showed the presence of two components with mobilities of -6.6×10^{-5} cm.^{2}/v./sec. and $-5\ 0\times10^{-5}$ cm ^{2}/v./sec. Eighty five per cent of the material was present in the more rapidly migrating component which was assumed to be the inhibitor. Since caeruloplasmin was present in these preparations, it appeared possible that this substance was related to the inhibitory activity.

Purification of hyaluronidase inhibitor by the authors and coworkers (unpublished data) showed that the inhibitor of human blood was precipitated in fractions II and III by method 6 of Cohn It was later demonstrated that part of the activity also was found in fraction IV–1. The inhibitor was precipitated in the combined fractions I, II and III by method 10 of Cohn, subjected to further alcohol fractionation, and finally precipitated by dilution to decrease ionic strength Preparations obtained in this manner were centrifuged in a preparative ultracentrifuge to remove a gel-like material which interfered with electrophoresis. Although such fractions showed only 30–35 times purification as compared with original plasma, they contained no detectible fraction migrating faster than about $-5\ 5\times10^{-5}$ cm ^{2}/v /sec upon electrophoresis in a 0 1 ionic strength veronal buffer, pH 8 6 After further fractionation by zone electrophoresis in a slab of potato starch, fractions, free of caeruloplasmin, with a purity of 600–700 times that of the original plasma were obtained Preliminary studies in free solution electrophoresis show only a single component with a mobility of $-5\ 4\times10^{-5}$ cm ^{2}/v /sec in 0.1 ionic strength veronal buffer of pH 8 6 Ultracentrifuge analysis, however, reveals small amounts of "impurities", which may be decomposition products of the unstable inhibitor

Chemical Nature

There is a general agreement that the non-specific inhibitor is heat labile (Haas, 1946, Dorfman, 1950, Wattenberg & Glick, 1952) and can be distinguished in this way from specific antibodies which are considerably more heat stable

The chemical nature of the hyaluronidase inhibitor remains unknown although its non-dialyzability, heat lability, sedimentation and mobility suggest that it is either a protein or closely associated with a protein in serum. Studies by Glick et al (1949) have indicated that the inhibitor is not identical with the serum mucoprotein of Winzler

Glick and coworkers have recently emphasized the relationship of hyaluronidase inhibitor to heparin As previously indicated, McClean (1942) pointed out that heparin is a hyaluronidase inhibitor, but that serum inhibition is probably not due to heparin because of the heat instability of the former. Glick & Sylvén (1951) concluded that the serum hyaluronidase inhibitor is a heparin-protein-lipid complex on the basis of circumstantial evidence, principally because a preparation of ox liver capsule (which is rich in mast cells) showed hyaluronidase inhibition In a subsequent paper Glick & Ochs (1952) reiterated this point of view because of a presumed parallel rise of hyaluronidase inhibitor and serum heparin (measured by metachromasia) in peptone shock, and prevention of this increase by previous india ink blockade. A rise of both heparin and hyaluronidase inhibitor following the administration of desoxycorticosterone acetate was also found These conclusions are open to criticism on both experimental and theoretical grounds. Careful examination of the data indicates that changes in inhibitor levels were small and no evidence of their significance is presented In addition, it seems dangerous to conclude that because two activities rise under a certain set of experimental conditions, they are identical It should be pointed out that the studies of Wattenberg & Glick (1952) showed no direct evidence of the occurrence of heparin in purified preparations of hyaluronidase inhibitor. Whereas it is entirely possible that the blood inhibitor may consist of some type of acid polysaccharide complex, present evidence does not prove the role of heparin.

The tissue source of the serum hyaluronidase inhibitor has thus far not been determined (Wattenberg & Glick, 1949).

Reaction with Enzyme

The nature of the reaction of the serum inhibitor with hyaluronidase has not been adequately elucidated since studies have not been conducted upon pure materials. Kinetic studies led Haas (1946) to conclude that the inactivation of hyaluronidase by serum is enzymatic. Differences of relative activities of sera of different species on hyaluronidase from different sources were interpreted as indicative of the presence of a series of physiologically important substances termed proinvasins and antiinvasins. On reinvestigation of this question, Dorfman et al (1948) questioned the validity of the method of calculation used by Haas and showed that the reaction between inhibitor and enzyme was not linear with time and is more rapid at lower

temperatures It was suggested that these facts were not consistent with an enzyme reaction, but rather with the properties of an inhibitor. Furthermore, the "proinvasin" activity postulated by Haas may be due solely to contamination of crude hyaluronidase preparations with proteolytic enzymes That serum inhibitor is not an enzyme which destroys hyaluronidase received further support from the studies of Hadidian & Pirie (1948) demonstrating that the reaction between inhibitor and enzyme is reversible, the reversibility being dependent upon ionic environment In subsequent studies Hadidian (1951, 1953) concluded that this reversibility is due to an enzyme present in certain hyaluronidase preparations which destroys the inhibitor A similar conclusion was reached by Werle & Moll (1950).

BIOLOGICAL STUDIES

During the last five years a large number of studies concerned with variations in the level of the serum hyaluronidase inhibitor have appeared In view of the fact that many of these have been repetitive only representative papers will be reviewed As has already been pointed out, the experimental determination of the hyaluronidase inhibitor presents many pitfalls Although the role of magnesium in the activity of the inhibitor has been clearly established, many of the published studies have not taken this fact into account in performing assays.

Most investigations have been concerned with the level of inhibitor in pathological states, although the physiological variations have as yet been imperfectly explored In a survey of levels of inhibitor in normal subjects, Dorfman et al (1948) found that serum levels in males between the ages of 16 and 45 years were lower than in females of this age This difference, although significant, was smaller than that found between normals and patients with disease states. The values obtained in children and adults over the age of 45 years showed less variation with sex. All population groups studied showed considerable variations within groups so that the above conclusions are valid only with respect to statistical significance of differences between means in large groups of individuals. The studies described above were carried out before the need for magnesium in the assay system was recognized. Repetition of these studies utilizing magnesium has led to identical results (Dorfman & Fried, unpublished results). Glick (1950) failed to confirm this sex difference in adults The reasons for this discrepancy are not obvious, but it should be pointed out that the

latter author expresses results as per cent inhibition and the studies of normals were carried out in the region of 20 per cent inhibition at which point the sensitivity of the method would be expected to be low. Hakanson & Glick (1949) found that the inhibitor level varied with menstrual cycle, being highest during menstruation, but the differences observed were small. No rise was observed during pregnancy, but a marked increase occurs in the immediate post-partum period. More recently, Ploman (1953) has failed to find any variation with menstrual cycle, but did observe an increase of inhibitor level in late pregnancy.

Good et al. (1951) have studied the relationship of levels of hyaluronidase inhibitor to adrenal function in the rat and concluded that the inhibitor level drops after adrenalectomy or hypophysectomy, being restored by the appropriate hormones. It was concluded that the rise of inhibitor in a variety of conditions represents a reaction to stress which is mediated by the adrenal It should be pointed out that variations upon which these conclusions are based are small, representing in some cases as little as differences of 5 per cent inhibition of enzyme

Relatively few studies have been conducted concerning the effect of nutrition on the level of serum hyaluronidase inhibitor. Tobin et al. (1948) found a fall in hyaluronidase inhibitor in protein depleted rats, while Schack et al. (1950) observed a small rise of inhibitor level in scorbutic *guinea pigs*

Several reports have been concerned with the levels of hyaluronidase inhibitor in acute rheumatic fever. Dorfman et al. (1949) found a marked increase in the level of inhibitor in young adults during the acute phase of rheumatic fever which later dropped to levels below normal and then returned to normal levels *It should be pointed out that these conclusions* depended upon statistical comparisons of the means of the groups studied with large variations within the groups Similar results were obtained by Good & Glick (1950), who found elevations in patients with acute streptococcal pharyngitis as well These authors also observed the drop below normal in convalescence, but believe that the level remains subnormal even after recovery Patients with Sydenham's chorea uncomplicated by other rheumatic manifestations showed no inhibitor elevation. Several studies have demonstrated that the elevated level in acute rheumatic fever is promptly depressed to normal or subnormal levels during treatment with ACTH or cortisone, (*Hakanson & Luft, 1949; Dorfman & Moses, 1950;* Adams et al. 1951). A similar depression of the elevated serum levels as

a result of treatment of a variety of other diseases has been reported by Faber & Schmith (1950).

Although a number of authors have postulated a special role for the hyaluronidase inhibitor in rheumatic fever it is now abundantly clear that the rise of inhibitor level has no specificity for rheumatic fever. Glick and his coworkers have studied the level of serum hyaluronidase inhibitor in a large number of diseases Most of these studies have been reviewed by Glick (1950) Significant increases of inhibitor level have been demonstrated in a variety of diseases including malignancies, virus infections, bacterial infections, rheumatic diseases and a number of diseases of unknown etiology This very wide range of conditions strongly suggests that this increase may represent a non-specific response to tissue inflammation or tissue destruction It might be thus classified with such reactions as elevated sedimentation rate, change in plasma proteins, etc.

Conclusions

The ubiquitous occurrence of the enzyme, hyaluronidase, and its striking effects on the ground substance of connective tissue has attracted considerable attention to its possible biological importance. This has led to concentration of attention on the inhibition of this enzyme in a search for the explanation of the mechanism of action of antirheumatic drugs and as a means of discovering new drugs It has been pointed out that there is as yet no definitive evidence to support the belief that hyaluronidase has any etiological relationship to rheumatic diseases It should be emphasized, however, that the general approach to this problem from the point of view of the metabolism of mucopolysaccharides may yield information of more immediate application The possibility exists that some of the inhibitors of hyaluronidase may have effects on other enzymes involved in hyaluronic acid metabolism, since inhibition probably depends upon chemical similarity to the substrate. Even in the case of certain low molecular weight compounds, existing evidence suggests inhibition of hyaluronidase depends upon polymerization or micelle formation

The existence of a serum inhibitor is of some interest but its biological function remains unknown It is apparent that the levels of this substance rise in a variety of diseases but the reasons and significance of these changes are not clear. Specific antibodies to hyaluronidases from various sources

have been well demonstrated and appear to be of some diagnostic value in complications of streptococcal infection such as acute rheumatic fever and acute glomerulonephritis.

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HORMONAL INFLUENCE ON CONNECTIVE TISSUE

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Introduction

A number of experimental and clinical investigations have revealed the decisive significance of the endocrine apparatus in maintaining the normal structure and function of the mesenchymal tissues. It has been shown also that administration of hormones may interfere with the course of a number of serious diseases that are reflected *inter alia* in the mesenchymal tissues

Hormones usually affect all three main components of the connective tissue, i e cells, fibrils, and ground substance. The intercellular substance is derived from and dependent on the cellular elements, influence on which may have profound effects on the structure of the supporting tissue

The following review on the hormonal influence on connective tissue will deal with experimental investigations of histological and chemical nature and with alterations in the structure and composition of the connective tissue during diseases of the endocrine system.

Experimental Investigations

The early studies on the hormonal influence on mesenchymal tissues were of purely morphological nature, using histochemical staining methods, later supplemented by autoradiographic examinations of connective tissue and studies of tissue cultures Parallel with the morphological studies, chemical analyses have been performed of various mesenchymal tissues.

Adrenocorticotrophin and Adrenal Cortical Hormones

The development of the adaptation theory by Selye (1946), the investigation into the pathophysiology of the influence of "stress" on the

organism, has provoked intensive studies on the "adaptive" hormones of the hypophysis and the adrenal cortex.

Adrenocorticotrophin The marked clinical effect of corticotrophin upon certain connective-tissue disorders gave the impetus to experimental studies of this hormone under normal conditions.

Corticotrophin *per se* probably exerts no effect on connective tissue. Its essential effect is that of mobilizing certain hormones from the adrenal cortex, primarily cortisone and hydrocortisone.

Cortisone Cortisone acts upon all components of the connective tissue, its cells, fibrils, and ground substance It has been well demonstrated by Castor & Baker (1950) in a study of the effects of prolonged local application to the skin of rats that the *fibroblasts* were materially reduced in number, and residual cells were often shrunken

The *mast cells*, which according to the studies of Asboe-Hansen (1950 a, b, 1952 a) appear to secrete the mesenchymal mucopolysaccharide hyaluronic acid, are also distinctly influenced by the administration of ACTH or cortisone His observations are based on histochemical studies of the dermal connective tissue of man, rabbits, guinea-pigs and mice treated with large doses of ACTH or cortisone. According to these investigations the connective tissue shows a material decrease in the number of demonstrable mast cells, and the residual cells appear more or less degranulated and usually somewhat smaller than normal. Asboe-Hansen used 4 per cent lead subacetate for fixation as well as freeze-dried material; he points out that alcoholic solutions are deleterious.

Other workers have observed the same inhibitory effect of cortisone on the mast cells (Stuart, 1951, Cavallero & Braccini, 1951, Bloom, 1952, Fulton & Maynard, 1953). Schoch & Glick (1953) were unable to confirm these findings and the same applies to Devitt, Pirozynski & Samuels (1953). These investigators performed their experiments on rats, using alcoholic solutions for fixation and staining Presumably, the divergent results are due to the use of different methods and animals.

In tissue cultures of embryonic chick skin Paff & Stewart (1953) demonstrated an inhibition of the activity of the mast cells while fibroblastic activity remained undisturbed.

Using autoradiographs with S^{35}, Asboe-Hansen (1953) studied dermal connective tissue from mice painted with the carcinogenic hydrocarbon 9,10-dimethyl-1,2-benzanthracene. By the stripping film technique he found distinct blackening over the numerous mast cells present in the induced skin

tumours There was, in addition, a diffuse, less intense blackening over the surrounding connective tissue. The explanation is that the labeled sulphate is taken up by the sulphuric mucopolysaccharides which occur mainly in the mast-cell granules, being less ample in the ground substance.

For comparison, he examined the tumour connective tissue from a group of cortisone-treated mice (1954). These specimens showed considerably less blackening over the mast cells, i.e they contained considerably less radioactive sulphur than the mast cells from the untreated mice. Over the ground substance, the blackening was only a little fainter.

Under the influence of cortisone, the mast cells become degranulated and lose sulphur. This effect of cortisone upon the mast cells proper is of essential importance. It shows that *cortisone acts upon the very source of important ground substance components* (Asboe-Hansen 1950 a, b, c).

These results accord well with Layton's (1951), who states that cortisone inhibits the incorporation of labeled inorganic sulphate, administered to experimental animals or to tissues *in vitro*, into chondroitin sulphate. Thus, he suggests that the action of cortisone on connective tissues is due to its inhibitory effect upon the synthesis of this mucopolysaccharide.

As mentioned above, there is also an inhibition of the fibroblasts that presumably take part in the formation of *collagen fibrils*. This inhibitory effect of cortisone has also been demonstrated in fibroblast cultures (de Lustig & Mancini, 1951) Following local application of cortisone to rat skin Castor & Baker (1950) found that the collagenous fibril bundles became arranged in an almost homogeneous compact mass, as a result of which the dermis was greatly thinned. The *elastic fibres* were apparently unaffected and therefore appeared more concentrated, as the ground substance was reduced in amount. In addition, all epithelial structures, including hair and sebaceous glands, underwent regression, and the subcutaneous fat disappeared almost completely.

W o u n d H e a l i n g. Already in the first clinical experiments with ACTH, Ragan, Grokoest & Boots (1949) observed a delay in healing of incised wounds and a depression of growth of granulations in open wounds.

By experimental investigations into the wound healing in normal animals, Ragan, Howes, Plotz, Meyer & Blunt (1949) demonstrated that the retardation of wound healing by cortisone involves a depressant action on many phases of the healing process, vascularization, deposition

of extra-cellular ground substance and formation of fibrils In the control animals, histological examination showed profuse growth of all elements of new connective-tissue, fibroblasts, blood vessels, and ground substance, whereas in the cortisone-treated rabbits all these elements were depressed.

This inhibitory effect of cortisone on granulation tissue is also reflected in a reduction in the oxygen consumption of the tissue (Scarpelli, Knouff & Angerer, 1953) The oxygen consumption of pooled dermal granulation tissue slices from rabbits were studied by the Warburg technique. These workers found in the cortisone-treated group a reduction of 66 per cent of the mean endogenous QO_2 value compared with untreated granulation tissue

The tensile strength of sutured wounds has also been tested, and by this method it was demonstrated that fibroplasia was delayed in rats under the influence of cortisone (Howes, Plotz, Blunt & Ragan, 1950).

It was demonstrated by Findlay & Howes (1952) that protein depletion produced by starvation facilitated the interference with wound healing by cortisone and conversely that the administration of cortisone accentuated impairment of healing due to protein depletion. As is well-known, administration of cortisone entails a negative nitrogen balance Such interference with the protein metabolism might be imagined to affect wound healing This is disproved, however, by the finding that *local application of adrenal cortical steroids* to experimental wounds interferes with the healing process (Baker & Whitaker, 1950) These investigators concluded that there was a direct action on the peripheral cells, especially fibroblasts and even on the interstitial matrix of connective tissue

The delaying effect of cortisone on wound healing of normal animals has not been demonstrable in all the investigations performed in this respect Marked differences in response have thus been found in different animal species As regards the effect of cortisone on wound repair, the mouse, guinea-pig, and rat are most resistant, more sensitive are the dog, the rabbit, and finally man (Ragan, 1952)

Fracture healing is also retarded following administration of ACTH and cortisone In experimental fractures, callus formation normally sets in after 4 days, being well-marked on the eighth day In cortisone-treated animals, on the other hand, the bleeding at the fractured site has not been absorbed and only faint signs of fibroplasia have appeared at eight days (Ragan et al, 1950).

Pleural and peritoneal adhesions. Attempts have been made to utilize the inhibitory effect of ACTH and cortisone on the formation of connective tissue in order to prevent the appearance of experimental pleural or peritoneal adhesions, induced by sprinkling the serosa with talc

The first experiments on the effect of ACTH on the appearance of peritoneal adhesions were published by Ducommun & Mach (1950), who reported a distinctly inhibitory effect in rat experiments In the dog Scheinberg & Saltzstein (1951) observed an inhibitory effect of cortisone on talc-induced peritoneal adhesions Similar findings have been reported by others In all these experiments, the inhibitory effect of cortisone on the development of peritoneal adhesions was convincing It is evident from the publications, however, that this requires cortisone in large doses, so large that it may entail systemic reactions, *inter alia* delayed wound healing.

In the reported experiments, the mechanism and point of attack of cortisone is the same as that mentioned under the wound healing experiments, viz inhibition of connective-tissue formation.

The inhibitory effect of cortisone on the development of tumours and on infection is discussed in the chapters by Simpson (p. 225) and Cavallero (p 214).

Spreading experiments It has been demonstrated that hormones which act upon the connective-tissue ground substance, also influence the spread of injected indicators. As early as 1940 it was pointed out by Menkin that adreno-cortical extract inhibits the spreading effect of testicular extract injected into the dermal connective tissue. As far as cortisone is concerned, this was shown by Opsahl (1949 b) by local as well as by systemic administration. A similar effect has been observed during administration of ACTH (Carstensen & Linderholm, 1952) Adrenalectomy leads to an increase of the spreading effect (Opsahl, 1949 a)

Many authors have interpreted this effect of ACTH and cortisone on the spread of injected dyes as a result of an inhibition of the action of hyaluronidase (Opsahl, 1949 a, b, Seifter, Baeder & Begany, 1949, Winter & Flataker, 1950)

This interpretation has been contradicted by Asboe-Hansen (1952 a) who states that the inhibition of the spreading of hyaluronidase in rabbit skin is due to a decrease and change in the hyaluronate in the connective-tissue ground substance following administration of ACTH or cortisone.

When the quantity and even the chemistry of the substrate on which hyaluronidase is to act has been altered its effect must also be reduced.

Hydrocortisone. Hydrocortisone or compound F (17-hydrocorticosterone-acetate) has been discovered in adrenal venous blood (Reich, Nelson & Zaffaroni, 1950) and in the peripheral blood (Savard, Kolff & Corcoran, 1952) These findings among others have led to the view that hydrocortisone is the principal hormone secreted by the adrenal cortex during ACTH stimulation.

It is quite apparent that most of the major metabolic and therapeutic actions of cortisone and hydrocortisone are qualitatively similar Hydrocortisone has been found to be more effective than cortisone The superiority of hydrocortisone was especially demonstrated after local administration to the skin and joints (Hollander, 1951)

In the rather insoluble acetate form, hydrocortisone exerts a pronounced topical effect without giving rise to generalized side effects (Conn et al, 1951). Zachariae (1954) demonstrated that hydrocortisone acetate, injected intraperitoneally in doses of 150 mg., distinctly inhibits the development of peritoneal adhesions in the rabbit induced by sprinkling the small intestine with talc Her experiments indicate that in this situation hydrocortisone acts in the same way as cortisone, inhibiting the formation of ground substance Zachariae & Moltke (1954) found local application of hydrocortisone to entail pronounced morphological changes of the numerous mast cells contained in the granulation tissue of experimental wounds in rabbit skin The mast cells were reduced in size, degranulated, and the metachromasia of the ground substance diminished These changes correspond to the findings in wounds, during systemic cortisone administration Hydrocortisone injected topically entails a regression of experimental skin tumours (Zachariae & Asboe-Hansen, 1954)

11-Desoxycorticosterone. This was the first adrenal cortical steroid to be synthesized (Steiger & Reichstein, 1937) Whether or not a hormone of this exact chemical composition is secreted by the normal human adrenal gland has been open to serious question Desoxycorticosterone exerts a pronounced effect on the electrolyte metabolism and is classified as a mineralo-corticoid

For experimental as well as clinical purposes, it is not the free desoxycorticosterone, but the acetate (DOCA) that is used The effect on the mesenchymal tissue has been studied particularly by Taubenhaus & Amromin (1949). They reported that prolonged treatment with DOCA resulted in considerable stimulation of the granulation tissue around tur-

pentine abscesses in the rat. Fibroblasts were large and bipolar, the collagen fibrils few and clumped together, whereas the ground substance was of a strange glassy appearance. When DOCA was injected during the development of the granulation tissue, no stimulation occurred (Taubenhaus & Amromin, 1950). DOCA is ineffective upon topical application, and it may counteract the inhibitory effect of cortisone on granulation tissue (Taubenhaus, Taylor & Morton, 1952). The same stimulating effect of DOCA on the proliferation of fibroblasts and the formation of intercellular material was observed by Pirani, Stepto & Sutherland (1951). They studied the granulation tissue of healing abdominal wounds in guinea-pigs—a method which seems preferable as the interaction of proteolytic and other enzymes from the large number of leukocytes of the turpentine abscess is avoided.

While DOCA stimulates granulation formation *in vivo* experiments by Cornman (1951) indicate that *in vitro* it inhibits fibroblastic proliferation

Growth Hormone (Somatotrophin)

Formation of granulation tissue has been found to be perceptibly reduced in hypophysectomized rats. During studies of the granulation tissue around abscesses induced by turpentine in the skin of rats Taubenhaus & Amromin (1950) observed that injection of growth hormone perceptibly increased the fibroblast proliferation and formation of collagen fibrils. The granulation tissue was abundant and exceeded that of the intact control animals

This finding is in accordance with Majarakis' (1946) observation of an increased tensile strength of wounds locally treated with growth hormone preparations

The stimulating effect of growth hormone on connective tissue is also noticeable in granulation tissue which has been under the inhibiting influence of cortisone (Taubenhaus, Taylor & Morton, 1952) The finer structure of such granulation tissue around turpentine abscesses showed normal organization and morphology.

Scow (1951) has demonstrated a stimulation of collagen formation in skin and muscle of thyroidectomized rats by the administration of growth hormone for 100 days.

THYROTROPHIN

In addition to stimulating the activity of the thyroid gland, the thyroid-stimulating hormone (TSH) of the adenohypophysis also exerts a direct effect on the connective tissue (Asboe-Hansen & Iversen, 1951)

Exophthalmos may be induced in the guinea-pig by injecting thyrotrophic hormone The histological changes of the retrobulbar tissue associated with such experimental exophthalmos bear striking resemblance to the pathological changes observed in human progressive or malignant exophthalmos (Smelser, 1937), *i e* violent oedematous swelling of the extraocular muscles and a marked increase in the volume of the connective tissue Microscopic examination shows lymphocytic infiltration and signs of connective-tissue proliferation (Naffziger, 1952)

In histochemical studies Asboe-Hansen & Iversen (1951) demonstrated accumulation of mast cells and mucopolysaccharide—particularly hyaluronic acid—in the retrobulbar tissue of guinea-pigs rendered exophthalmic by thyrotrophic hormone Ludwig, Boas & Soffer (1950) also found an increase in hyaluronic acid In addition to the histochemical demonstration, the latter workers also studied the hexosamine content of the retrobulbar tissue following hydrolysis and found an increase This also indicates that mucopolysaccharide has accumulated in the retrobulbar tissue (hexosamine is a hydrolytic product of mucopolysaccharides)

Accumulation of the water-binding hyaluronic acid in the retrobulbar tissue perhaps explains the increase in the volume of the orbital tissue and the resulting exophthalmos

Furthermore, it has been demonstrated that injection of thyrotrophic hormone in the guinea-pig is rapidly followed by a transport of fat from the normal depots to the muscles, liver, and kidneys. These studies have even shown hyperlipæmia and phagocytized droplets of fat in the circulating leukocytes (Dobyns, 1946, Asboe-Hansen & Iversen, 1951)

The accumulation of mucopolysaccharides observed in experimental exophthalmos is most marked in the retrobulbar connective tissue, but it has also been noted in the peripheral muscles, and in the perirenal, axillary, and peritesticular regions (Asboe-Hansen & Iversen, 1951)

These changes occur in intact as well as thyroidectomized animals, but are most marked in the latter. In other words, the tissue reactions must be interpreted as a result of a *direct* effect of the thyrotrophic hormone on the tissues.

Thyroxin inhibits the thyroid-stimulating and exophthalmic effect (Smelser, 1938) as well as the fat-mobilizing effect (Iversen & Asboe-Hansen, 1952) of thyrotrophin.

According to these investigations, the effect of thyrotrophin on the connective tissue is opposite to that of cortisone. Cortisone exerts a distinctly inhibitory effect on the various elements of the connective tissue, cells as well as ground substance, whereas thyrotrophin has a stimulating effect. In addition to the changes in the ground substance, viz. an increase in hyaluronic acid, there is also an increase in the number of mast cells in the connective tissue

This phenomenon has also been elucidated by spreading experiments, injecting hyaluronidase into the skin of thyroidectomized rabbits (Iversen & Asboe-Hansen, 1953) After 10 days' treatment with thyrotrophin, the spreading effect of hyaluronidase proved to be considerably increased This is explained by an increased amount of hyaluronate in the dermal connective tissue The hyaluronidase is thus given more substrate to act upon, and this makes for a marked difference between the spread in normal and in thyrotrophin-treated skin.

It is the same phenomenon that is operative in human myxoedematous skin in which the spreading of hyaluronidase is perceptibly increased compared with normal skin (Asboe-Hansen, 1951).

Thyroxin

Nothing is known about the direct point of attack of thyroxin in the tissue. Administration of thyroxin to normal animals in doses that render them thyrotoxic leads to alterations in the dermal connective tissue. The ground substance stains only faintly or not metachromatically with *toluidine blue, only a few mast cells are visible, predominantly* in the perivascular areas (Asboe-Hansen, 1950 c)

Similarly, thyroxin considerably increases the subcutaneous spread of hæmoglobin (Ducommun, Timiras & Dordoni, 1951).

Sex Hormones

Oestrogen. Certain monkeys develop at puberty changes in the skin surrounding the genitals The skin and underlying tissues swell, assuming a

reddish purple colour. It was demonstrated by Allen (1927) that the sex skin changes in the macaque monkey are dependent on the ovaries The changes cease after ovariectomy and can be reactivated by injection of an oestrogen Treatment of immature macaque monkeys, males or females, with this hormone induces the same changes as in natural conditions (Zuckerman, von Wagenen & Gardiner, 1938) The material that so abundantly accumulates in the skin under hormonal stimulation was identified as hyaluronic acid by Chain & Duthie (1940) The sex skin is an extremely firm tissue, not pitting under pressure, but it was demonstrated by Duran-Reynals, Bunting & von Wagenen (1950) that inoculation of hyaluronidase into the sex skin brings about a softening of the tissue These investigators found, by histochemical studies of the sex skin injected *in vivo* with hyaluronidase, a complete disappearance of the metachromatic reaction with toluidine blue Similar results have been reported by Taylor & Sprunt (1943), who found oestrogens to inhibit the spread in the skin of rabbits. Lurie (1950) demonstrated that oestrogen retards the progress of tuberculosis at the point of inoculation in the skin and diminishes its dissemination to the internal organs in rabbits, chiefly by reducing the permeability of the connective tissue In certain animals the oestrogen increases the turgidity of the connective tissue elements by increasing the intercellular fluid, which presumably is connected with increased formation of hyaluronic acid (Sprunt, 1950) Hereby the spread of particulate matter in the skin is inhibited

This presumably corresponds to the variations found in woman during the menstrual cycle Women often become somewhat oedematous immediately before and during the early menstrual period At this juncture, the excretion of oestrogens in the urine increases They gain weight, and the daily output of urine is decreased during these days Ploman (1953) has studied the dermal absorption by means of a photoelectric dermofluorometer registering the disappearance of an intradermally injected indicator, a uranin solution He found that women in the beginning of menstruation have a somewhat faster dermal absorption than those tested one week later in the cycle Women in an advanced pregnancy exhibited a considerably accelerated uranin disappearance

Relaxin In addition to the systemic changes of the connective tissue, pregnancy is also associated with topical changes, e g in the symphysis pubis Under the influence of the hormone of pregnancy *relaxin* (Hisaw, 1925), proliferation of the connective tissue in the symphysis pubis occurs during

pregnancy, *i.e.* separation and relaxation of the symphysis takes place (Talmage, 1947). It has been demonstrated by Perl & Catchpole (1950) that the ground substance in the pubic regions of guinea-pigs which had been treated with relaxin or which were in advanced stages of pregnancy undergoes changes which are believed to be depolymerizing in nature. The connective tissue stained more deeply with the McManus-Hotchkiss method (cf. p. 33), the fibrillar elements proved looser, and intravenously administered Evans blue became localized in these regions. The tissues assume a more fluid consistency and a certain degree of mobility is attained.

Testosterone. The growth of the cock's comb has long been known to be dependent upon stimulation by androgens (Hardesty, 1931) Ludwig & Boas (1950) have confirmed these earlier investigations by using a pure male hormone, testosterone. They found that the increase in comb size varied directly with the dose of the testosterone administered. In the combs from the testosterone-treated cockerels they found the structure of the connective tissue much looser and less compact; the collagen bundles were widely separated by large amounts of amorphous interfibrillar and intercellular material which stained metachromatically with toluidine blue. The metachromasia was totally removed by digestion with bull testis hyaluronidase. From these results they suggested that a considerable proportion of the material is hyaluronic acid Boas (1949) has extracted from such tissue a pure substance which was chemically identified as hyaluronic acid In the combs from the testosterone treated animals the fibroblasts were more numerous than in the controls and were considerably larger due to abundant, strongly basophilic and pyroninophilic cytoplasm.

In another paper Boas & Ludwig (1950) reported that the administration of the α-oestradiol to immature chicks prevents the normal growth and development of the comb Microscopical examination of the combs of oestrogen-treated birds revealed the failure to deposit the metachromatic interstitial ground substance normally seen.

α-oestradiol failed to diminish the comb-growth response produced by testosterone and gonadotrophins, indicating that oestrogenic inhibition of comb growth is dependent on the suppression of gonadotrophin secretion in the adenohypophysis

Endocrine Disorders Involving Changes in the Mesenchymal Tissues

A number of endocrine disorders are reflected *inter alia* in the connective tissue. That some of these diseases involved connective-tissue changes had been known long before it was realized that the connective tissue was anything but passive supportive tissue It was not until much later that the connective tissue was recognized as an active and dynamic tissue with its own, specific functions

Thyroid Diseases

Myxoedema It has been known for years that hypothyroid conditions may be accompanied by accumulations of a mucinous substance in the dermal connective tissue Horsley (1885) was one of the first who reported an accumulation of mucin in the connective tissue of myxoedematous humans and monkeys

A similar accumulation of mucin occurs in localized, circumscribed myxoedema This condition is most common following subtotal thyroidectomy for thyrotoxicosis, and in such cases it is often associated with exophthalmos Carol (1932) was the first investigator who chemically demonstrated such a mucin-like substance in the skin in localized myxoedema, a substance that reduces Fehling's solution following hydrolysis with hydrochloric acid

The mucinous substance is made up predominantly of mucopolysaccharides bound to proteins. These mucopolysaccharides are hyaluronic acid and chondroitin sulphuric acid (Watson, 1946, Watson & Pearce, 1947)

By histochemical methods many investigators (Unna, 1894, Kreibich, 1927, Reuter, 1931) have demonstrated accumulations of mucinous substances in myxoedematous skin Asboe-Hansen (1950 c, 1951) has histochemically confirmed Watson and Pearce's finding of increased hyaluronic acid and chondroitin sulphuric acid

The histological appearance of the dermal connective tissue in myxoedema is extremely characteristic The mucin stains metachromatically with toluidine blue in lead subacetate-fixed preparations, and there are accumulations of mast cells in the dermal connective tissue For details reference is given to the chapter by Asboe-Hansen, p. 274

Myxoedema is nearly always accompanied by a fairly marked accumulation of water in the tissues, presumably in the main due to the water-binding capacity of hyaluronic acid (Ropes, Robertson, Rossmeisl, Peabody & Bauer, 1947)

As is well-known, the myxoedematous state is reversible, the tissue changes yielding to administration of thyroxin as the clinical condition improves During treatment with thyroxin, the mast cells change from large and well-granulated into small, degranulated cells. The metachromasia of the ground substance will subside or entirely disappear just as the bursting apart of the collagen fibrils will rapidly decrease (Asboe-Hansen, 1950 c).

The above histological changes in the dermal connective tissue are present in varying degrees in hypothyroidism, also where myxoedema is not evident In adults, the diagnosis of hypothyroidism or myxoedema is not difficult, determination of the B M R affords a further support. In children, on the other hand, it may be extremely difficult to make a diagnosis of congenital myxoedema, inter alia because determinations of the basal metabolic rate may be rather cumbersome and difficult to interpret.

Microscopical examination of the dermal connective tissue of children with congenital myxoedema has proved of a certain diagnostic significance Andersen, Asboe-Hansen & Quaade (1954) examined 47 children with definite or suspected hypothyroidism. The histological findings in the skin biopsies proved to be a criterion that is positive in more than half the children with definite hypothyroidism and negative in children whose thyroid function is not reduced. In a group of children for whom the *diagnosis of hypothyroidism was in doubt, the skin biopsies were positive* in almost half the cases and thus supported the diagnosis which was confirmed by adequate thyroxin therapy. This study showed that a negative skin biopsy could not rule out a diagnosis of hypothyroidism. In collaboration with Wichmann the authors (1954) found high thyrotrophin values *in the blood serum of the patients who exhibited positive skin biopsies*

Thyrotoxicosis. In dermal connective tissue from patients with *thyrotoxicosis* the ground substance stains only faintly metachromatically with toluidine blue. This faint metachromasia occurs only in the papillary layer; only a few mast cells are visible, mainly of a perivascular habitat (Asboe-*Hansen, 1950 c)*

Although myxoedema is cured by thyroxin, this is not tantamount to

the condition being due to a deficiency of the thyroid hormone proper. Certain findings indicate that thyroid insufficiency causes a preponderance of the thyrotrophic anterior pituitary hormone and that this is the cause of the myxoedematous changes. Normally, there is a certain balance between the thyroid gland and the hypophysis (thyroid-pituitary axis), the thyroid-stimulating hormone (TSH) stimulating the thyroid gland to increased production of thyroid hormone (TH), which in return inhibits the pituitary production of TSH

Completely or partially abolished thyroid function, therefore, disturbs this balance, resulting in a preponderance of the anterior pituitary hormone

Malignant Exophthalmos An increased thyrotrophin content in the blood plasma has been observed in *malignant, progressive exophthalmos* (De Robertis, 1948, Purves & Griesbach, 1949, d'Angelo, Paschkis, Gordon & Cantarow, 1951, Asboe-Hansen, Iversen & Wichmann, 1952) This is one of the reasons why the thyrotrophic hormone has been attributed with an important role in the pathogenesis of malignant or progressive exophthalmos

This condition is often associated with localized myxoedema, usually affecting the prætibial region This localized myxoedema shows accumulation of mucopolysaccharide and a resulting binding of water in the connective tissue It would be reasonable to suppose that similar changes favouring the accumulation of mucin-like substances were present in the retrobulbar tissue of patients with malignant exophthalmos. In experimental thyrotrophic exophthalmos in guinea-pigs an accumulation of mucopolysaccharides, particularly hyaluronic acid, has been found in the retrobulbar connective tissue by Asboe-Hansen & Iversen (1951), who believe that the water-binding capacity of hyaluronic acid is responsible for the oedema of the retrobulbar space and also for the development of exophthalmos in the animals

In addition to the changes mentioned above, the experimental animals treated with thyrotrophin exhibited considerable accumulation of fat in the skeletal muscles—extra-ocular as well as peripheral. This accumulation of fat in the muscles and the subsequent degeneration of the contractile elements were so pronounced that they urged histochemical studies of the skeletal muscles of patients with progressive, malignant exophthalmos

The study was carried out by Asboe-Hansen, Iversen & Wichmann (1953), who obtained biopsy specimens from the quadriceps femoris and the biceps brachii from patients suffering from malignant exophthalmos

and other thyroid-pituitary disorders (thyrotoxicosis, myxoedema, and acromegaly). No or very little fat was found in the muscles. But in the preparations fixed in 4 per cent lead subacetate muscular changes predominated the microscopic picture. Following staining with toluidine blue,



Fig 1

Photomicrograph Transverse section of skeletal muscle fibres from a patient with malignant exophthalmos Staining Toluidine blue Note metachromatic "half-moons" between sarcolemma and fibres, peripheral nuclei are located at the inside of the "half-moons". (G Asboe-Hansen, K Iversen & R Wichmann Acta endocrinol *11*, 376, 1952)

there were metachromatic, homogeneous masses within the sarcolemma, but outside the muscular substance proper. In transverse sections they appeared as semilunar masses, in longitudinal sections spindle-shaped This substance was susceptible to hyaluronidase, and its polysaccharide character was further confirmed by Hale's method, in which iron is bound to the acid polysaccharides in the tissues and then demonstrated *in loco* with potassium ferrocyanide, that stained the masses Prussian blue. By the periodic acid-Schiff (PAS) method the half moons stained an intense red Stained with hæmatoxylin-eosin they were practically invisible and by the van Gieson-Hansen method they stained faintly yellow. Formalin-fixed preparations showed no such masses. Upon very close inspection, however, their presence was indicated by empty spaces within a "loose-fitting" sarcolemma.

In other words, there are intrasarcolemmic accumulations of a hyaluronidase-sensitive, acid mucopolysaccharide Electron microscopy confirmed that these accumulations are within the sarcolemma, outside the contractile substance (Iversen, Asboe-Hansen & Carlsen, 1953)

The muscular changes were found in 10 out of 10 examined cases of malignant exophthalmos, in 16 out of 45 cases of thyrotoxicosis, in 1 out of 7 cases of myxoedema, in all 5 examined cases of acromegaly, and in none of 25 patients without pituitary-thyroid disorders. These findings are believed to be related to the muscular weakness which is often a predominant symptom of these diseases The phenomena were not observed in the experimental animals treated with thyrotrophin

The thyrotrophin content of these patients' sera was determined by a biological method, using as an indicator the height of the thyroid cells of starved tadpoles of Xenopus laevis according to a method developed by d'Angelo, Paschkis, Gordon & Cantarow (1951) and modified by Wichmann

In 10 out of 11 patients with malignant exophthalmos the thyrotrophin level was significantly increased

These muscular changes occurred in various peripheral skeletal muscles They must, accordingly, be presumed to represent a widespread, probably generalized muscular lesion

The findings support the theory that progressive exophthalmos is of thyrotrophic genesis

The fact that the muscular changes occurred in all the patients with progressive exophthalmos and in about one-third of the patients with thyrotoxicosis indicates that these two lesions are genetically related and that progressive exophthalmos is merely a special manifestation of thyrotoxicosis. It is evident that malignant exophthalmos may be ranged as a pituitary disease just as well as a thyroid disease

PITUITARY DISEASES

Morbid changes in the anterior hypophysis may give rise to dysfunction of its hormone production causing increased or decreased secretion of one or several of the superior hormones Such dysfunction results in a number of characteristic lesions that may involve the mesenchymal tissues

Acromegaly To-day it is generally accepted that acromegaly and gigantism are due to increased secretory activity of the eosinophil cells

in the adenohypophysis. The most common cause is eosinophil adenoma The eosinophil cells in the anterior lobe are secretory cells that are believed to produce the growth hormone (somatotrophic factor). Thus, mice suffering from hereditary dwarfism have no eosinophil cells in the adenohypophysis (Smith & MacDowell, 1931).

The clinical picture of acromegaly is well-known. Practically all tissues and organs are involved in the morbid process that is characterized by generalized hyperplasia and hypertrophy. It involves the skin, muscles, interstitial connective tissue, and mucous membranes. For details concerning the histological picture of the tissues involved, reference is given to the chapter by Asboe-Hansen, p. 289

In acromegalic subjects, the incidence of tumours is higher than normal, presumably because of the accelerating effect of somatotrophin on tumour development. Most of the tumours are benign, usually skin fibromas Another characteristic feature is the high frequency of keloids.

Simmond's Disease. This disease is due to hypofunction of the adenohypophysis with consecutive pluriglandular insufficiency. In advanced cases, the patients develop pituitary cachexia with senile changes of tissues and organs. They present a picture of premature senility, with dry, thin, and wrinkled skin with reduced elasticity, hairing is scanty, the pubic and axillary hairing disappearing entirely and the supercilia being thinned out laterally.

Adrenocortical Diseases

Cortisone has a distinctly inhibitory effect on the various elements of the mesenchymal tissues This is particularly evident in the condition characterized by hypercorticoidism

Cushing's Syndrome. This disorder is due to hyperfunction of the adrenal cortex that may be a consequence of hyperplasia or tumour of the cortex. It may also be a phenomenon secondary to hyperfunction of the adenohypophysis or due to a tumour in the anterior lobe. Such a tumour is usually a basophil adenoma, but sometimes the syndrome may be caused by chromophobe and acidophil adenomas which by exerting mechanical pressure stimulate the basophil cells to increased secretion of ACTH that again induces increased activity of the adrenal cortex. Lastly, many symptoms of Cushing's syndrome may be induced by prolonged treatment with ACTH or cortisone.

It is an increased production of glucocorticoids by the adrenal cortex that is directly responsible for the symptoms.

The patients present a characteristic appearance of enormous obesity, particularly localized to the face, neck, and trunk. They exhibit virilization with hypertrichosis and the skin undergoes typical changes. The clinical picture and the histology of the various mesenchymal tissues involved are described by Asboe-Hansen, p. 290.

Addison's Disease. Insufficiency of the adrenal cortex—as seen in Addison's disease—is accompanied by typical pigmentation of the skin and mucous membranes. The pigmentation is due to deposition of melanin that is present in the epidermal cells—particularly the basal ones—and in the cells of the corium. The pigment occurs particularly in cicatricial tissue and in areas that are pigmented beforehand, such as e.g. the areola of the breast, the genital and anal mucosa. The mechanism of pigmentation is entirely obscure.

In the endocrine disorders discussed above, the hormonal influence on the connective tissue is evident and generally accepted. In a number of other endocrine diseases, the connective-tissue reactions are still little known or have not been studied. It is worth mentioning, however, that the complications of longstanding *diabetes* are associated with accumulations of mucopolysaccharides in the renal glomeruli and in the retinal microaneurisms.

Hormonal influence on the connective tissue is not manifest only in disorders of the endocrine system, but also in a number of diseases reflected in the mesenchymal tissues that cannot—at our present stage of knowledge—be related to the endocrine system. We need only recall the effects of ACTH and cortisone upon diseases such as rheumatoid arthritis and periarteritis nodosa. Processes as fundamental as inflammation—bacterial as well as allergic—are influenced by the administration of hormones as apparent from various chapters of this book. The development of tumours may also be influenced—accelerated or inhibited—by various hormones. These findings, mainly derived from experimental investigations, have already been utilized in clinical practice.

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AGING OF CONNECTIVE TISSUE

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IN REVIEWING the age changes in connective tissue it is convenient to consider them under three headings: collagen and reticulum, elastic tissue; and ground substance The age changes in bone and teeth will not be considered.

Collagen and Reticulum

Observations by Bucciante & Lauria (1934) indicate an increase in the percentage of collagen in striated, non cardiac, muscle as age increases. Using chemical analyses, Myers & Lang (1946) found that after the age of 50 the proportion of collagen in the thoracic aorta began to rise. Harman & Webster (1949) analyzed 34 normal human hearts for total and alkali soluble proteins, collagen and elastin In the ages from birth to 80 years they found no differences in the proportions of these constituents from samples of ventricles, but there was an increase in the proportion of collagen in the atria of some individuals over 10 years of age.

Degenerative changes in the collagen of the skin of aged individuals such as hyalinization, fragmentation, and reduction in number of nuclei were described by Goldzieher (1946) Ejiri (1936) noted an atrophy and gradual disappearance of collagen fibers in the skin at an advanced age Vohwinkel (1931), in studying the skin of the face, found degenerative changes in collagen beginning at about the 4th decade These changes resembled those caused by X-ray and radium On the other hand, Hill & Montgomery (1940) believed that, since no changes were present in skin from protected parts of the body in individuals over 60 years old, the alteration in the collagen described by previous authors was the result of exposure rather than a direct result of aging. They did, however, describe the collagen fibers in the skin of the aged as elongated and stretched out.

In the normal development of collagen in the skin and tendon of animals, the number of fibroblasts decreases in proportion to the number of collagen bundles from the young to the adult (Ceresa, 1936, Ingelmark, 1948, and Andrew, 1951)

In embryonic tissues, the early appearance of the white connective tissue is in the form of a reticular network which stains black with the silver stains in contrast to collagen which stains brown (Wolfe et al. 1942) In rats there is a marked increase in the proportion of collagen to reticulum in the skin between 20 to 35 days after birth and in the tail tendon 16 days after birth (Gross, 1950 b, Randall et al. 1952).

Maximow (1928) noted this same phenomenon in tissue cultures of fibroblasts Here a reticulum was first laid down followed in 30 to 50 days by the gradual appearance of thicker bundles of fibers which had lost their argyrophilia and stained brown with silver This reticulum was given the name "precollagen" by Schwarz & von Pahlke (1953) although "precollagenous reticulum" would seem to be a better term. For some time it was thought that the precollagenous reticulum and collagen were essentially the same The difference in silver staining was ascribed only to a physical difference in that reticulum had a narrower fiber than the collagen. Recently, however, von Pahlke (1954) made electron microscopic examinations of collagen from human fetal, baby, juvenile and adult tendons stained with the Gomori and the periodic silver tetramine techniques He found that in the fetal stages silver particles were scattered irregularly on the surface of the fibrils (Fig. 1 a and b). As development proceeded, there was a shifting to a more regular periodic grouping of silver particles In addition to this surface deposit, other fibrils had a periodic inside banding (Fig 1 c) This latter type of fibril increased in number as age increased so that it made up the majority of the fibrils in juvenile and mature Achilles tendons. Von Pahlke concluded that the fibrils with banding on the inside were mature collagen and appeared brown in the light microscope while fibrils with a different type of silver staining were immature and appeared black Since these techniques liberate aldehyde groups to which the silver attaches, this may indicate a change of chemical structure in the collagen during the development of the mature fibril. The work of Schwarz (1953) indicates that the type of silver staining does not depend upon the width of the collagen fibril since both types of silver deposit can be found in fibrils of the same width.

Heringa & Weidinger (1941–42) first reported an X-ray diffraction

pattern characteristic of amorphous material for the precollagenous reticulum in newborn rat skin. Between 20 to 30 days after birth, a collagen pattern developed as the ratio of collagen to reticulum increased Gross



Fig 1

a and *b* Achilles tendon collagen from 17 cm fetus, formalin fixed and stained by the Gomori silver technique Silver is deposited irregularly in globules on the surface of the fibrils (× 21600) (Courtesy Dr Gunter von Pahlke, Freie Universitat, Berlin)
c Achilles tendon collagen from 13 year old child Formalin fixed, and stained by the Gömori silver technique Silver is deposited in regular bands within the fibrils (× 21600) (Courtesy Dr Gunter von Pahlke, Freie Universitat, Berlin)

(1950 a) in collaboration with Bear, found that the amorphous X-ray pattern obtained from the skin of the newborn rat was completely eliminated by treating the tissue first with trypsin They attributed the results of Heringa & Weidinger, therefore, to the large amount of ground substance in the skin of the newborn Feitelberg et al (1949) examined the chordae tendinae from human hearts at differing ages and found a poor orientation of the "collagen molecules" below the age of 4 years and an



Fig. 2
Collagen fibrils with tapered ends Formalin fixed Palladium shadowed (× 18,700)

increasing degree of orientation above this age. All specimens over 45 years of age had a high degree of orientation.

Gross (1950 b) using the skin of the rat, von Pahlke (1954) using the human Achilles tendon, and Schwarz (1953) using human sclera showed an increase in width of the collagen fibrils as age increased. The widths became stabilized at 60 days in the rat skin, by at least 13 years in the human Achilles tendon, and in the 7th to 8th month of fetal development in the case of the sclera. The corneal fibrils, however, remained in their embryonic state (Schwarz, 1953). During the fetal stages of development of the human Achilles tendon there was a predominance of a single fibril width with only a narrow variation. At 1¾ years the variation in width increased considerably and there was no single predominant width In tissue cultures of fibroblasts Porter (1951) observed an increase in collagen fibril width as the cultures grew older. He postulated a deposition of molecular collagen on the surface of the fibrils after their initial formation.

An estimation of relative collagen fibril length is possible with the electron microscope. Natural fibril ends can be recognized since they are smoothly tapered rather than broken (Fig. 2). If many natural ends are seen in a certain sample, it can be deduced that the fibrils are shorter than in a sample where few tapered ends are found. Banfield (1952 a) has shown that shorter fibrils are abundant in embryos and scarce in adult skin

indicating, at least in the early stages of development, an increase in fibril length as well as width as age increases Randall et al (1952) noted an abundance of tapered collagen fibrils in the umbilical cord of a 10 day embryo rat and Porter & Vanamee (1949) described them in tissue cultures of fibroblasts

Though von Pahlke (1954) reported only a 640 Å periodicity in human Achilles tendon collagen and precollagenous reticulum, fibrils with a shorter periodicity have been described by Randall et al. (1952) in rat tail tendon and by Porter & Vanamee (1949) in tissue cultures. These were thought by the latter authors to represent an early stage of collagen development The fibrils had a periodicity of 270 Å and, as the age of the culture increased, a characteristic mature collagen periodicity of 650–800 Å developed by a variable thickening of the 270 Å bands in groups of three Table 1 summarizes the differences between the immature and mature collagen fibril

TABLE 1

Comparison between mature and immature collagen fibrils

Immature Fibril	*Mature Fibril*
Fibrils short	Fibrils longer
Fibrils narrow	Fibrils wider
270 Å repeating pattern	640 Å repeating pattern
Black with silver stains	Brown with silver stains
Silver deposited irregularly on surface of fibril	Silver deposited as a stain within the fibrils bringing out the mature periodicity
Amorphous X-ray diffraction pattern	Oriented X-ray diffraction pattern with 640 Å periodicity

In addition to the morphological changes in collagen during development there are other alterations with age that can be shown by enzymatic and physico-chemical methods Nageotte (1927) demonstrated that the collagen of rat tail tendon could be dissolved in dilute acetic acid and subsequently reconstituted by precipitating with sodium chloride It was also shown (Nageotte & Guyon, 1934) that the collagen from young animals was more soluble than that from old In an extension of this work, Banfield (1952 b) tested the acid solubility of human abdominal skin taken postmortem from a large number of individuals ranging in age from the premature infant to the very old. The skin was extracted with 1 : 1000 dilution of acetic acid under constant experimental conditions Sodium chloride was added to the extract and the precipitate which formed was

graded 0 to 4+ It was found that the skin of all individuals below one year of age yielded soluble collagen and that the extract from 50 per cent of them gave a 4+ precipitate However, above one year of age there was a certain percentage of individuals whose skin did not yield soluble collagen and from none was the high solubility indicated by a 4+ precipitate obtained.

This same procedure was carried out on dura and Achilles tendon. Again, all samples from individuals below 1 year of age yielded soluble collagen Above this age soluble collagen was obtained only once from the tendon of a 5 year old The results for dura above 1 year of age were more variable.

Jordon-Lloyd & Marriott (1935) measured the swelling at various pH's of tail tendons from young (6 week) rats and one old rat. Above a pH of 2, the tendons of the old rat swelled to a greater extent than those of young rats. Below pH 2, both old and young tendons swelled to the same degree The reason for this was thought to be the breaking up, below the pH of 2, of the constricting reticular sheath which surrounds the collagen fibers of the tail tendon Above a pH of 2, however, the reticular sheath of the young animals was more resistant to the action of the acid, thus resulting in less swelling of the collagen fibers

Banfield (unpublished) found essentially the same thing for human Achilles tendon from the younger age group There was an increase in swelling in 1:1000 dilution of acetic acid up to the age of 28 years After this age, however, swelling decreased to the age of 50 after which the tendons remained virtually unswollen

Keech (1954) followed the hydrolysis by collagenase of skin collagen from individuals of different ages. To determine the amount of digestion, she measured the increase in nitrogen in the supernatant fluid by the

TABLE 2

Summary of differences between young and old collagenous tissues

Young Collagenous Tissue	*Old Collagenous Tissue*
Swells in 1:1000 dilution of acetic acid	Decreased amount of swelling in 1:1000 dilution of acetic acid
Soluble in 1:1000 acetic acid	Decreased solubility in 1:1000 acetic acid
Susceptible to the action of collagenase	Decreased susceptibility to the action of collagenase
Large number of fibroblasts	Smaller number of fibroblasts

Kjeldahl method. The results showed that the abdominal skin collagen from infants under 1 year of age was twice as susceptible to the action of collagenase as the adult, and that the skin collagen of children from 1 to 10 years of age was 1½ times as susceptible Table 2 gives a comparison between young and old collagenous tissues

Collagen has been considered to be relatively inert metabolically. This has been confirmed by studies using labelled glycine, which indicate that once collagen is laid down its turnover is extremely slow (Neuberger & Slack, 1953).

Elastic Tissue

Elastic fibers of the skin, during the process of aging, undergo fragmentation, clumping, and acquire an affinity for basic stains These changes generally begin between 30 and 40 years and are confined to skin over exposed parts of the body (Schmidt, 1891, Vohwinkel, 1931, Hill et al, 1940, Dick, 1947) The number of elastic fibers in the dermis decreases with age (Lindholm, 1931) Those in the papillary layer are absent under the age of 5 but by the age of 20 they are abundant (Dick, 1947)

Known age changes in the elastic tissue of the aorta are summed up by Lansing (1951) in the second conference on connective tissues sponsored by the Josiah Macy, Jr. Foundation He described a fraying, fragmentation and granulation of the elastic lamella and the acquisition of a marked affinity for calcium These changes begin at about 20 years and are more marked in the abdominal than in the thoracic aorta There are also differences demonstrable by dye uptake In the old elastic tissue there is a greater affinity for resorcin and orcein, the old elastic tissue is stained by hematoxylin whereas the young is not, and old elastin stains yellow-orange while the young stains red with congo red Chemical analysis of elastin from the abdominal aorta showed an increase in the aspartic and glutamic acids above the age of 55 without significant change in glycine, proline, leucine, isoleucine or valine The increase in aspartic and glutamic acids was not observed in samples of elastin from the pulmonary artery. The calcium content of the aorta increased greatly even though atheromatous areas were avoided but there was only a moderate increase in the calcium content of the pulmonary artery.

In the coronary arteries beginning at 20 years a progressive fragmentation of elastic interna was observed

Ground Substance

There is almost no information concerning age changes in the ground substance except that it is more abundant in the infant than in the adult (Bensley, 1934, Day, 1947, Gross, 1950 a, Bunting, 1953).

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THE CONNECTIVE TISSUES IN WOUND HEALING

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Introduction

In the lower forms of animal life, whole organs or limbs may be regenerated after wounding In the salamander, for example, an entire limb may be regenerated. Man has no such capacity for regeneration of lost parts after wounding but he does possess a remarkable capacity to restore continuity of the injured area. To do this, only three tissues are regenerated during wound healing; blood vessels, fibrous tissue and epithelium. These three tissues comprise the scar or cicatrix The processes that produce them are termed endothelial proliferation, fibroplasia and epithelization An abundant new growth of blood vessels is essential for the regeneration of the other two tissues and precedes slightly Epithelization restores surfaces, either squamous or mucous, while fibroplasia restores the continuity of deeper structures The regeneration of these three tissues form the scar or cicatrix All three tissues however, are differentiated after their production to reach the mature state that they maintain ultimately in the scar.

In general all the deep tissues wounded are healed by fibroplasia. The wounded derma of the skin, muscle, fat and preexisting connective tissues are reunited by a cicatrix of connective tissue. The development of the connective tissue scar blocks the regeneration of muscle, fat, nerves and the epithelium of organs, hence the statement "cicatrization blocks regeneration" On the other hand, the connective tissue cicatrix begins the restoration and return of function of wounded connective tissues and their derivatives cartilage and bone

Proliferation of the mesenchymal tissue begins three or four days after wounding, after the inflammatory or exudative phase of healing This process of fibroplasia can be seen on the third day microscopically and

grossly on the fourth day The granulations that appear in the depths of the open wound on the fourth day consists of sprouting new capillaries surrounded by mesenchymal tissue, proliferating by the process of fibroplasia Thereafter, the process is most active in the next seven to nine days.

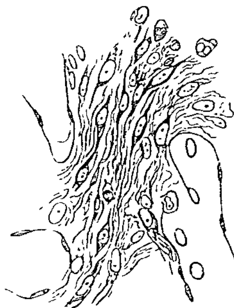


Fig 1

Seven day Wound Young capillaries of granulations surrounded by numerous fibroblasts Sparse fibrils of connective tissue

In the first two of these, the fibroblasts multiply, but the entire length of time is occupied with the deposition of fibrils of collagen between the cells. These fibrils are oriented around the new vessels at first (Fig. 1), but with the passage of time, their concentration becomes more dense (Fig. 2). After two weeks of healing they are still of fine size and they run in many directions They are still undifferentiated and their pattern resembles the embryonal. Blood vessels are still numerous between the fibrils and although there are fewer cells per unit of area than on the seventh day of healing, many are present.

The origin of the fibrils has long been a matter of controversy. Previously, attempts were made to solve the question of their origins by morphological comparisons. The idea of transformation of plasma clots (Baitsell, 1946) or fibrin fibrils (Nageotte et al, 1930) to connective tissue fibres as judged by staining reactions have now been replaced by the

more exact knowledge about the chemistry and structure of the collagen fibre. It is known the fibre contains a mucopolysaccharide (Meyer et al, 1937) and has definite spacings seen by the electron microscope (Schmitt,



Fig 2

Fourteen day Wound Blood vessels smaller Fibroblasts diminished in number per unit of area Many fine connective tissue fibrils have appeared with some parallel arrangement but still oriented about the blood vessels

1942) and that it originates either as an extending process from fibroblasts or in juxtaposition to the fibroblast Porter et al (1949) has described the formation of collagen fibres in tissue cultures of fibroblasts.

Origins of Generated Connective Tissue

Fibroplasia begins with the appearance and multiplication of mononuclear cells and fibroblasts. These cells are brought to the site of injury during the preceeding exudative phase of healing through the new growing

capillaries that begin to sprout about the third day. These fibroblasts also come from nests about blood vessels and from the loose connective tissue of the body. The differentiated connective tissues of the body cannot dedifferentiate to give origin to young cells that start the process of elaborating new connective tissue after injury.

This is an extremely important concept, for it means that fascia, ligaments, the heavy connective tissues, bone and tendon and the derma of the skin and all mature collagenous structures and their derivatives cannot give origin to new connective tissues after injury. Instead a group of mobile cells coming from the bone marrow, the reticulo-endothelial system or embryonal nests are the sources of supply. Radioactive studies have confirmed these findings for it can be shown that the mature connective tissues of the body do not metabolize rapidly (Kellgren, 1952) and in them change takes place slowly.

During the seven to nine days of fibroplasia, the strength of the wound rises and then plateaus off (Fig. 3) As judged by the rate of ascent of strength, the process of fibroplasia takes place at the same rate in such diversified anatomical structures as muscle, fat and fascia. It has the same rate too in all the laboratory animals and in man (Fig. 3). One is startled to find for example, the same rate for the process in the healing of the wound of the mouse, with its greatly increased metabolic rate, as in the healing of the wound of man

Finally, the strength acquired after two weeks of healing is the same per unit of area in the wounds of different tissues and in different laboratory animals

Strength of the Wound and Fibroplasia

Because they are the strongest tissues and do not necrose as readily as the others, the connective tissues in either cut edge of the wound are the structures to sutures. When they are properly sutured they give the greatest immediate strength to the sutured wound (Howes, 1940).

After two weeks of healing, the relationship of the strength of the scar to the original strength of the skin wounded can be illustrated by considering the healing strengths of wounds of skins in different laboratory animals. At this time, at the end of the period of fibroplasia, the normal strength of the skin is not regained by any of the wounds. In the rat 13 per cent of strength is restored, in the rabbit 9 per cent, in the cat

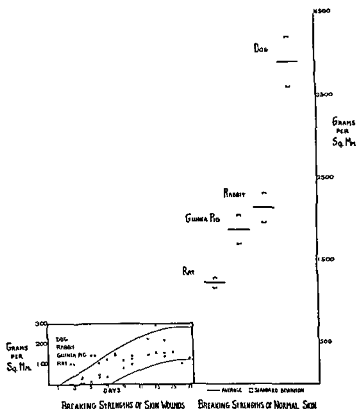


Fig 3

Note that on the left, the strength of fibroplasia is rising at approximately the same strength in all four animals

2 per cent and in the dog 5 per cent (Fig. 3). In general less of the original strength is regained in the larger animals. The differences however are occasioned by differences in the original strengths of the skins wounded rather than by differences in the strength of the cicatrices (Fig. 4). The stronger skins are found in the larger animals. The collagen fibres of the corium of the uninjured skin are large and well differentiated according to structure They are practically of the same size in each animal, the corium is thicker, however, and there are more of them in the large animals thereby giving the greater strength (Howes et al, 1939)

The tissue of the cicatrices on the other hand has relatively the same

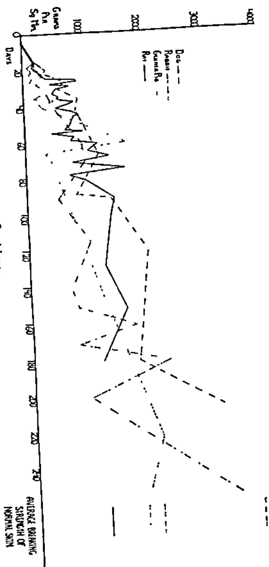


Fig 4

Period of Fibroplasia is the heavy black line on the left Period of Differentiation extends from end of fibroplasia until the breaking reaches the original strength

strength per unit of area; corresponding to the relatively undifferentiated pseudoembryonal pattern of the generated connective tissue.

The data suggest that the process of fibroplasia acting in response to injury is independent of the tissue or the species of animal involved and is accordingly governed by its own laws. What is known about these laws will be discussed later

In tissue like muscle, fat and the walls of the gastrointestinal tract, the generated connective tissue of the early scar is stronger than the surrounding tissues

Usually the size of the scar ultimately resulting from wounding is smaller than the size of the original wound Contraction of the wound accounts for this reduction in size Philosophically this would suggest that scar tissue is a poor substitute for the original tissue wounded, for fibroplasia occurs abundantly and extensively at first and then the excess of connective tissue is reabsorbed

The Size of the Cicatrix

Contraction begins with the gross appearance of granulations in the depth of the open wound This powerful force that reduces the amount of cicatrix to be generated, acts for about two weeks following its initiation Its rate has been plotted by Du Nuoy (1932) Starting with maximum intensity, the sides of the defect approach each other and then its rate gradually tapers off

There are certain areas of the body where contraction does not occur and the scar is as big as the original wound These places are where the soft tissues are in a single layer over bone as for example in the scalp, over the patella and the back of the hand. Contraction is influenced by the tension lines existing in the tissues injured so that the final shape of the scar is pulled in the direction of these lines

This new connective tissue is laid down abundantly, not only between the cut edges but layers are bound together by it If infection develops, more tissue is destroyed and the cicatrix becomes even more abundant The excess of the abundant cicatrix is partially absorbed during the period of differentiation of the scar. This absorption that proceeds along with differentiation takes six months to a year It follows Wolff's law that states regenerated and transplanted tissues continue to exist only in response to function If function is interfered with, then differentiation and absorption

does not occur. Thus if there has been an infection and a mass of scar tissue results, function of the part is destroyed, the bulk of undifferentiated scar tissue remains and in turn function does not return. *In other words,* over-production of scar tissue interferes with the return of function and a lack of function allows scar tissue to remain undifferentiated. Healing, that is to return function to the wounded part, should ideally produce only an amount of cicatrix consistent with uniting the continuity of the injured part.

Differentiation

Differentiation of the newly generated connective tissue in the scar is a protracted process and is variable depending on the tissue and other special circumstances where the cicatrix is located.

In the healing of cartilage and bone, as soon as the pseudoembryonal pattern of connective tissue is laid down, calcium salts are deposited and cartilaginous cells appear. If cartilage is to be regenerated this may be the end point of the process of repair save for an increase in the size of the collagenous fibres and their orientation in a direction to meet stress

If bone is to be regenerated, then an undifferentiated ossification occurs with a disappearance of the cartilaginous cells. Later this primary callus is differentiated and cortex bone forms with absorption of great masses of the primary callus. Considerable stress is laid on the function of osterclasts in the realignment but the fact is overlooked that the connective tissue of the primary callus is also reabsorbed and in the final formation of the cortex bone heavy collagenous fibres are formed and they also are realigned to meet the exergencies of stress. No special cells are ever given credit for the reorientation of the connective tissues in the healing bone.

In the soft tissue scar, the fibrils present after two weeks of healing thicken They become fewer in number per unit of area of cicatrix. Fibroblasts decrease in number per unit of area along with the number of blood vessels. The new scar that was red with its abundant capillaries immediately after healing becomes white with the disappearance of blood vessels. Disappearance of the blood vessels occurs also in exposed granulations. For this reason granulations two months old will not accept skin grafting as readily as freshly formed granulations.

Immediately after continuity of the wound is restored the fibrils of the cicatrix are oriented in a whirling fashion about the new capillaries but



Fig 5
Collagen fibres of derma
Guinea pig × 200

Fig 5 a
Collagen fibres of derma
Rat × 200

as the fibres thicken, they become rearranged in response to function and take on the pattern of the original connective tissue wounded.

For example, Zinn (1927) showed that if a square of fascia lata was excised, after two weeks of healing this square was entirely filled with a white matrix through which the muscle beneath could be seen. Microscopically this new tissue was composed of fine fibrils of generated connective tissue running in all directions and many blood vessels The surface of the square was partially adherent to surrounding structures. When a similar defect was inspected after one year of healing, the tissues in the defect could not be distinguished from the surrounding fascia lata, save for the markings at the corners of the square It was a dense white The surface was no longer adherent and microscopically large bundles of collagen were found, and the majority of the heavy fibres ran in a parallel direction similar to those in the original fascia lata The differentiation that had occurred during the year had completely rearranged the primary connective tissue cicatrix to a pattern of connective tissue resembling the original tissue

In the healing of the skin the differentiated collagen fibres of the uninjured derma may be seen in Figs 5 and 5 a Some of the large fibres run in all directions so that in the photographs some are cut transversely. In Figs 6, 7, 8 the differentiation of the fibres may be seen that took place with the long secondary increase in strength Fig 6 shows the pseudo-embryonal pattern just after fibroplasia while Figs 7 and 8 show the increase in size of the fibres with differentiation At first, in Fig. 7 most of the fibres run parallel and only later in Fig 8 a can fibres be seen running in another direction (Howes et al, 1939)

When a tendon is wounded as early as eight days the fibrils begin to align themselves parallel to the original ones of the tendon This early



Fig 6
Connective tissue regenerated by fibroplasia, 14 days Guinea pig × 200

Fig 6a
Connective tissue regenerated by fibroplasia; 14 days Rat × 200



Fig 7
Connective tissue healing, 51 days Rat × 200

orientation in response to function has led to the belief that there are special "tendoblasts" that give origin to these parallel early thin fibrils However the notion is lost when the process is observed in the healing of a tendon attached to a paralyzed muscle, for then no differentiation occurs and the cicatrix remains in the pseudoembryonal state with fine fibres running in all directions. However as soon as the muscle begins to function again the parallel realignment occurs

The process of differentiation of the connective tissue scar never completely reforms the original structure injured (Fig. 9). The elastic and nerve fibres that grow back in are very rudimentary. The scar of the tendon can always be identified microscopically from the original tendon injured, and the bone of the callus is always somewhat different.

Hyperdifferentiation of the collagen fibre occurs in keloid formation. Very large ones form but in addition there is a persistance of some of the mononuclear cells that are usually found in the early day of fibroplasia.

In the recovering of surfaces, the growth of granulations exerts a reciprocal relationship to epithelization and mesothelization. New squamous



Fig 8
Connective tissue healing; 82 days
Rat × 200

Fig 8 a
Connective tissue healing, 91 days
Guinea pig × 200 Note some of
the fibres seen cut in cross section

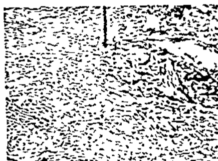


Fig 9
Wound Rat's skin 158 days × 95 On the left the connective tissue of the scar and on the right the connective tissue of the original derma particularly at the top Note that many of the fibres of the scar are almost as large as those of the derma, that they are packed more densely and they never seem to stain as deeply as the fibres of the original derma

or mucous epithelia will not extend across granulations that are depressed nor will they grow over protuberant granulations Granulations must be just at the right height to allow the epithelial cells to spread across the surface Conversely however, this spread of epithelial cells across the granulations stops further outward growth The mechanisms that govern these reciprocal relationships are poorly understood

In the peritoneal or pleural cavities or in joints—mesothelial cells will rapidly cover all free surfaces However if two denuded areas become approximated by fibrin and then granulations extend between them, a fibrous scar results and the serosal recovering fails Such is the mechanism of formation of adhesions in intestinal, pleural and serosal cavities

Factors and Laws Influencing Fibroplasia

These may be divided into local factors within the wound and general ones concerned with the well being of the wounded host. Save for the influence of age, all detract from the rate of fibroplasia indicating that the process may be interfered with more readily than it can be stimulated

Infection in the wound, hematomata, the presence of excessive amounts of foreign bodies, crushed tissue and interference with blood supply, all delay the onset of the process of fibroplasia. Infection is the chief offender particularly in the early, invasive, necrotizing stage. The last four mentioned conditions predispose towards infection but even by themselves they can retard the onset of the process. On the other hand, as soon as the infection localizes or becomes chronic, fibroplasia takes place about its periphery, and only the pocketing of pus and the persistence of slough keeps the granulations from extending into these areas. Unlike the circumstance at the onset of the process, the presence of an abundant number of bacteria in the area does not seem to be a deterrent to the process of fibroplasia.

The general factors all except one, also delay the onset of fibroplasia after wounding. A deficiency of vitamin C has a specific effect. The fibroblasts appear and some rudimentary fibrils, but the fibrils do not increase in density nor do they ever increase in size. Microscopically the delay suggests a cortisone effect. The concentration of vitamin C in the tissues wounded during the repair is the important determining factor and not a temporary change in the blood level Lund et al. (1946) have shown that as much as 500 mg. of vitamin C a day may be required for an injured individual to obtain tissue saturation.

All metabolites and proteins are mobilized to the site of the wound in the first 48 hours after its infliction. Sodium, potassium and calcium are concentrated there and the injured area will borrow the needed substances from the normal tissues. The depletion must be very severe therefore to affect the reparative processes, but fibroplasia is the more sensitive of the three. A low serum protein delays the onset of fibroplasia (Thompson et al., 1938). If an animal is starved and given a low protein diet until 20 per cent of its original weight is lost, fibroplasia either does not occur or is definitely slowed (Findlay & Howes, 1952). Combinations of protein and vitamin C deficiencies work together to retard fibroplasia. There is no statistical evidence that the retardation caused by metabolic deficiencies occurs through lowering the resistance of the wound to infection even though

infections are more prevalent in experimental animals. Depletion of potassium salts has not been found to influence the process (Findlay & Howes, 1950). Under experimental conditions, realimentation quickly restores the capacity of the animal for fibroplasia, yet clinically in chronically debilitated states—in chronic ulcerative colitis, in advanced tuberculosis and in diseases that affect the liver, where fibroplasia is retarded—the process is not always restored by realimentation

In young animals, fibroplasia definitely starts a day earlier than in the adult (Howes & Harvey, 1932) There is no proof however, that the time of onset or the rate is slowed in the aged. Anemia (Sandblom, 1944) and dehydration (Bird & McKay, 1932) slow the onset of fibroplasia Excessive bleeding occurs in the wound and healing is delayed when there is a deficiency of Vitamin K.

Effects of Hormones on Fibroplasia

Both the adrenocorticotrophic hormone of the pituitary gland (ACTH) and compound F found in the adrenal gland have been shown to delay the appearances of granulations in the healing wound. In 1949, Ragan Grokoest & Boots reported that ACTH retarded healing of biopsy wounds made in clinic patients Eight years earlier, Cuthbertson et al (1941) and Mueller & Graham (1942) were not able to show that anterior pituitary extract had any effect on the rate of healing of wounds in animals Thus it appears that either humans are more sensitive to ACTH or the previous workers were using impotent extracts. Later in 1949, cortisone became available in larger amounts, and Ragan, Howes, Plotz, Meyer, Blunt & Lattes demonstrated that after its parenteral administration, the appearance of granulations was delayed in the healing wound of the rabbit These same authors found that with an adequate parenteral dose of cortisone, blood clots at fracture sites were not resorbed as rapidly. Still later that same year, Howes, Plotz, Blunt & Ragan showed that the dose of cortisone given parenterally was crucial in determining whether the appearance of granulations would be delayed Large doses repressed their appearance while smaller doses did not (Table 1). Even a small amount of cortisone employed locally would delay the appearance of granulations. Microscopic sections of wounds treated with cortisone, showed that new blood vessels did not sprout, fibroblasts did not proliferate and reticulin was not deposited (Fig 10). In Table 1, it will be noted that even with

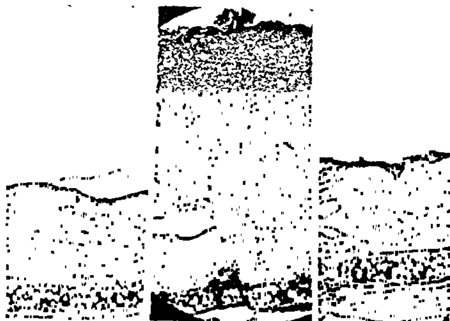


Fig 10

Cross sections of normal rabbit's ear, *left*, 8-day granulating wound, control, *center*, and 8-day granulating wound, animal treated with cortisone, *right* Note thickness of connective tissue and the cell density in the normal ear. In the control wound, the granulations are higher, there are many fibroblasts with collagen fibrils, and many capillaries are seen Two old large dilated blood vessels are present The cortisone ear is not much thicker than the normal connective tissue of the ear and all the old large blood vessels are dilated There are no new capillaries However, the cell concentration, particularly of the round type and those resembling fibroblasts, is increased. These cells show few signs of increased proliferation There is less exudate on the surface than on the control granulations

the largest dose of cortisone, the appearance of the granulations was only delayed for 18 days and that thereafter granulations grew. With smaller doses they appeared sooner and grew. Thus the greatest delay was less than three weeks or stated conversely granulations grew after three weeks despite the administration of the largest amount of cortisone. The corollary is, and this is the important point, cortisone given parenterally never completely obliterates fibroplasia, it only puts off its appearance. Later Meyer showed that if a small particle of cortisone was placed on the conjunctiva of an irritated eye, blood vessels would dilate and sprout but not in the area where the cortisone was located.

Clinical confirmation about the dose came quickly. Thus it was found

TABLE 1

Parenteral Dose—Rabbit (Mg per kg per day)	*Results*
8 to 10 mg per kilogram	Complete cessation of growth of granulation until 18 days
5 to 6 mg per kilogram	Same results for 12 days
3 6 mg per kilogram	Onset of fibroplasia 9th day
2 0 to 2 3 mg per kilogram	Onset of fibroplasia delayed 7–8 days

that wounds gave no gross evidence of disturbed healing when hernia patients received small doses of 2 mg. per kg. cortisone—the usual dose used for arthritis.

Investigations to explain the action of cortisone were soon undertaken. Cortisone sets up an "alarm reaction" disturbing mineral metabolism Sodium is witheld and potassium is excreted Protein is lost in the urine. Protein catabolism occurs and protein synthesis is depressed. Fibroplasia proceeds with protein synthesis and protein anabolism Glycogen is stored in the liver Edema of the tissues is lessened. Mast cells along the blood vessels are increased in number and become vacuolated Weight loss is not striking A masking analgesia occurs Local action of cortisone however, would have to be explained on the basis of its peculiar character as a steroid compound

Clinical trials were carried out in an attempt to utilize the capacity of cortisone to distort the growth of granulations Some of these were patterned after the laboratory findings while others disregarded them and encompassed the complete range of pathology of the connective tissue and its healing An example of the latter was the employment of cortisone for the treatment of keloids Cortisone was shown to delay the onset of fibroplasia but there was no evidence that it would keep large collagen fibres from forming once they had appeared Hence it was not surprising that in one clinical experience keloids actually formed under the influence of cortisone and was not prevented in others The use of cortisone locally however, in the reconstruction of joints was well founded on the possibility of utilizing the delay in the appearance of granulations Stenchfield (1950) who did the original work in animals still feels that he obtains improved clinical results with cortisone used locally with oxycel for this purpose

The results obtained with cortisone to prevent postoperative adhesions have been contradictory As stated, intestinal adhesions represent an

abundance of fibrous cicatrizations occurring where mesothelial regeneration did not take place As yet, it is not known whether proliferation of the mesothelial cells is also repressed by the action of cortisone. Lymphatic cells are certainly reduced in number by cortisone. Hence allergic response to transplanted homologous tissues, can be delayed for a short time.

The possibility of obtaining poor healing in patients receiving cortisone for the treatment of another disease should be discussed. In general, the therapeutic levels used clinically, are not high enough to delay the onset of the formation of granulations unless the patient's serum proteins are considerably below normal (Findlay & Howes, 1952). Usually, the patient has taken the cortisone for some time also, so that the anticortisone factors that offset the inhibiting effect are already mobilized, and granulations will grow as they do in laboratory animals after receiving cortisone for three weeks

Lastly, when cortisone is withdrawn in the laboratory animal receiving an inhibitory dose, granulations again appear within four days. Healing begins after the usual lag period beginning on the day when cortisone is withdrawn In the patient however, rapid withdrawal cannot be carried out because of the danger of adrenal failure The adrenals are usually atrophied after a long continued administration of cortisone, and therefore the dose should be reduced gradually and some ACTH should be given also to stimulate the adrenals to secrete again.

It must be remembered that healing in all patients takes place in the presence of some circulating cortisone. This amount apparently does not account for the length of the exudative phase of healing, for the length of this period is not reduced when the adrenals are removed.

Lastly, some remarks should be made about the influence of ACTH on fibroplasia. Despite the earliest clinical findings that ACTH delayed the healing of biopsy wounds, the laboratory experiments did not show repression of the formation of granulations until extremely large doses were employed, these amounts greatly exceeding any used clinically. However, the clinical patients were sick and a possible explanation may be found in our work showing that even small doses of cortisone interfere with healing when the serum proteins are low and the animal is malnourished. (Findlay & Howes, 1952). ACTH must act by causing a secretion of compound F from the adrenals It is doubtful that in the normal individual enough F can be produced by stimulation with ACTH to distort the onset of fibroplasia.

Transplantation of Connective Tissues

Many connective tissues are being transplanted in modern surgery and the fate and survival of these grafts must be understood as part of the healing process. Bone, cornea, cartilage and skin, that is largely derma, are now used from so called "banks". The notion that tissues separated from their blood supply for any length of time but stored in some special way, in serum, in the deep freeze, after lyophilization or by treatment with some form of electrical or radiation emergency are "living grafts" seems to surround many of these bank activities.

To remove tissues from one location to another in the body, the cells must survive until they obtain a new suitable nutritional environment. Cells vary in their capacity to survive when separated from their original blood supply. They also vary in their nutritional requirements not only individually but if they are proliferating and growing versus proliferating only for the purpose of replacement of cells that die It is important to realize that all cells separated from their blood supply for a sufficient length of time, die and even in the body they have a life span and must be replaced. The idea of a "living graft" is always relative then and certainly if a tissue is to survive and proliferate after transplantations it must receive a new blood supply. These are the conditions for autogenous grafting. In homologous grafting a complicating factor arises, namely, the resistance of the host to these foreign tissues, resulting in thrombosis of the blood supply after varying lengths of time and finally death of the graft

The fifty year old demonstrations that tissues of the mesenchyme can be transplanted autogenously, homologously or fresh or stored in any form and that they seemingly regain their shape and become incorporated into the tissues were proven to be true again and again by Nageotte, Ollier, Phemister and many others They found them to be replaced by "creeping replacement" after a long period of time. At first, a new blood supply was observed entering transplanted connective tissues, and later new mesenchymal tissues were deposited and differentiated until the structure resembled the original. Whether the new structure continued to persist depended on function. Thus a dried piece of tendon placed on the liver swelled, and a month later looked like transplanted tendon, but after a year only a scar remained. It was absorbed because it had no function. On the other hand, when a similar piece of dried tendon was sewed between the ends of a cut tendon, again it took on the appearance of a transplanted

tendon after a short period of time, but two years later was still there In this instance it continued to exist in response to function (Wolf's law)

Several unique circumstances are found in the transplantation of connective tissues that give the impression they may be living grafts, even when they are transplanted homologously or in preserved states.

1) There are relatively few cells in differentiated connective tissues. The collagen fibres and the mineral salts are byproducts, so to speak, maintained in a living mobile state. The cells can die and disappear and grossly the greater mass of tissue is still intact and appears unchanged unless it is allowed to dry. Like all other tissues, the connective tissues die when deprived of blood supply but the duration of the time they will survive is not exactly known. With our present histological methods, manifestations of death in the fibres are not apparent and changes in the cells are difficult to make out because they are relatively scarce in the large matrix of collagen and it is not generally realized that they are the components that must be studied to distinguish whether connective tissues are living

2) Dead differentiated connective tissue requires the action of a special enzyme to bring about its solution. A true collagenase is required to dissolve collagen unless it is very denatured. Wounded tissues are devoid of collagenases so that a long process of denaturation must occur before dissolution begins. Thus the transplanted connective tissue is first encapsulated like any foreign body, the blood supply grows into its interstices and then slowly, disintegration and redisposition goes on.

Transplanted connective tissue or connective tissue arranged as a pedicle tends to squeeze out its newly inhabiting blood supplies after a year This change sometimes destroys the intended function of pedicles made of connective tissue. For example, pedicle flaps arranged to improve collateral circulation around the damaged liver or in hydrocephalus or in coronary disease have always failed because the blood supply was ultimately squeezed out by the cicatrix.

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THE PATHOLOGY OF CONNECTIVE TISSUE

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Space is not available to give more than a sketchy outline of what we consider some of the more pertinent observations in relation to certain lesions that occur in connective tissue. Since this is not intended to be a complete review of the subject numerous significant papers have not been mentioned.

We have intentionally avoided any detailed morphologic descriptions of alterations in specific diseases because they are readily available in the current literature It seemed advisable that instead we should stress the pathogenesis of some of the more general reactions as they occur in connective tissue

Because of the ubiquity of connective tissue and the frequency of its participation in pathologic processes, it is not surprising that its reactions should be an object of extensive investigation at present As studies progress, a clearer picture of connective tissue capabilities and reactivity is emerging.

It is the ambition of this presentation to provide an attitude toward degenerative diseases of connective tissue which, we hope, will allow a certain correlation of clinical, pathological and experimental data As will become apparent, there are many gaps in the information available, but for the purposes of continuity and clarity an overall picture is attempted It is conservative to say, we think, that exciting and stimulating possibilities for the obtainment of new knowledge exist at the present time.

The interaction of an injurious agent and living tissue results in a histologic picture that characterizes a disease process Since it is possible to prevent disease in some instances by the elimination of the injurious agents, bacteria and viruses for example, clinicians naturally, and other investigators have been preoccupied by these causative factors This has led, gener-

ally, to insufficient consideration of tissue reaction or potentialities This is particularly true in relation to diseases of the connective tissue

It has become increasingly clear that many connective tissue changes formerly considered specific are, in reality, an expression of the limitation of the connective tissue response to injury For example, Klinge (1933) believed that fibrinoid formation was diagnostic of a hyperergic state. Since the tissue change may be observed, however, in a number of lesions in which the element of hypersensitivity may be ruled out (bases of peptic ulcers, cysts, bursae and ganglia, e g.), fibrinoid has lost that diagnostic specificity.

Klinge's work was valuable nevertheless in that he clearly recognized the significance of Schade's (1923) previously described concept of the "connective tissue system" to pathology This desirable feature divorced from the allergic implications was retained by the proposal of the concept of *diffuse collagen disease* (Klemperer, Pollack & Baehr, 1942) However, "even this cautious synthesis was premature because it resulted in an indiscriminate acceptance of a term with a diagnostic and pathogenetic import not originally intended when it was conceived" (Klemperer, 1950)

The arguments stated above with regard to the hyperergic implication of fibrinoid may also be applied to other degenerative lesions of the connective tissue Basic similarities in the pathogenesis of many of these lesions certainly do exist and have led some authors to believe that some sort of etiologic relationship exists between diseases characterized by sclerosis or by amyloid, hyaline or fibrinoid formation (Leupold, 1925, Loeschke, 1927, Knežević, 1944) If we realized clearly, however, the non-specific character of these tissue reactions and the limitations of the connective tissue response, we would be spared that unproved, premature and probably inaccurate conclusion

This non-specific nature of the connective tissue reaction raises the question of the relative value of approaching the problem of degenerative disease of the connective tissue from an etiologic point of view It is entirely within the realm of possibility that many etiologic agents may participate in creating a described abnormality Thus, it is possible that hypersensitivity reactions, cold, anoxia, etc, may all contribute their share toward the development of arteriosclerosis and it may be more rewarding to be more seriously concerned with the basic pathogenetic mechanisms rather than possible specific etiologic features.

The histologic studies of the degenerative lesions of the connective tissue referred to above are valuable in that they have established the com-

mon features of the processes. An interstitial edema which is relatively cell free and non-metachromatic, followed by a mucinous accumulation are the characteristics shared These findings, too, indicate the applicability of Rössle's theory of serous inflammation (Rössle, 1943) at least as it applies to the connective tissue. This idea refers to certain phenomena affecting

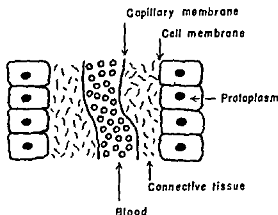


Fig. 1.

the hemato-parenchymal barrier (Fig. 1). Non-specific damage to any of the three anatomic components (endothelium, connective tissue or parenchyma) may precipitate a series of events which include (a) increased capillary permeability, (b) decreased tissue permeation, (c) impaired tissue respiration (partial anaerobiasis), (d) increased tissue content of sodium, chloride and water, (e) decreased tissue content of potassium and phosphate and (f) increased tissue mucopolysaccharide content in subacute lesions. (Altshuler & Angevine, 1951, Eppinger, 1949, Eppinger et al, 1935).

Various authors have stressed the importance of each of the morphologic components in the pathogenesis of the tissue lesions. It must be remembered that accurate temporal relationships have not been established, as yet, so that real evaluation of these claims is not possible. In other words, the primarily affected anatomic element is unknown in any given lesion.

As stated above, many agents may induce these tissue changes. Bacteria, viruses, toxins, heat, cold and hypersensitivity reactions are only some of the pathologic influences. Desoxycorticosterone, testosterone, estrogens, thyrotropic hormones are some "physiologic" influences which may have a simi-

lar effect (Altshuler & Angevine, 1951) The pathologic process, then, may be exaggeration of a normal tissue response For this reason, the term "serous inflammation" may not be altogether desirable and has been criticized (Aschoff, 1938)

Since the alterative and reactive tissue processes may vary in severity and chronicity, observable lesions differ. In the milder forms edema alone is noted, whereas in the more severe varieties, necrobiotic alterations of connective tissue and blood vessels as well as of the parenchymal cells may occur. Following mild transient injury, complete restitution may ensue, while lesions of longer duration ultimately result in sclerosis of blood vessels and connective tissue.

It is apparent from the above illustrations of the irritative mechanisms that some may be characterized by a generalized connective tissue effect while others are of only local significance Diabetes, myxedema and hypersensitivity reactions are illustrations of generalized forms Local abnormality, in part, is explainable by limited application of the damaging agent (cold to extremity, e g). In some instances, however, special tissue susceptibility must be postulated When androgens are administered to a caponized cock, for example, the increase in size of the comb is due to a "serous inflammatory" alteration and is accompanied by an accumulation of metachromatic material (Hardesty, 1931, Boas, 1949, Ludwig & Boas, 1950). The reaction in the comb, of course, is out of proportion to the response in other tissues.

Since many agents may have this generalized effect upon the connective tissue, it is apparent that the term, "diffuse collagen disease", must not be interpreted as indicating an etiologic relationship between various disease states so characterized In this more general sense, diabetes and myxedema might also lay claim for inclusion amongst these diseases and are certainly different in etiology from the rheumatic diseases

Other corollaries follow recognition of this basic connective tissue process For example, it has been fashionable in the past several years to make much of the so-called "general adaptation syndrome" Promulgated by Selye (1946), the concept refers to the damage induced in experimental animals by chronic exposure to trauma The trauma is nonspecific in character but the damage is believed to be mediated through persistent and excessive secretion of desoxycorticosterone-like hormones The connective tissue lesions thus developed in the heart, synovia and blood vessels, to select a few, are said to be highly suggestive, in turn, of the

Aschoff body of rheumatic fever, the synovial lesions of rheumatoid arthritis and the vascular lesions of periarteritis nodosa.

The published pictures of the histologic sections, however, are not convincing of their similarity to human lesions and, even if they were, they would not necessarily establish this as the human pathogenic mechanism Until it can be shown that sufficient numbers of patients with these disease processes are actually excreting clinically significant quantities of desoxycorticosterone-like substances, definitive conclusions are not warranted. Such evidence is lacking. The sweat-salt excretion tests, as indicating the quantity of desoxycorticosterone-like hormone(s) produced, have not been impressive

It is difficult also to reconcile some of Selye's own experimental findings with his conclusions For example, he found that if animals were given large doses of desoxycorticosterone over a period of time, he could expect a certain number of connective tissue lesions If he damaged the animal by other means simultaneously, he potentiated the development of these lesions If the pathogenetic mechanism were truly through the adrenal cortex alone in the manner postulated, why would exposure to cold or fatigue increase the number and severity of the connective tissue lesions described? *It would seem more logical to refer all these influences to* the hematoparenchymal arena primarily.

In a similar sense, the fact that certain drugs may alleviate clinical symptomology does not necessarily indicate that they are of consequence with regard to etiologic considerations. It is possible, obviously, to affect certain aspects of the pathologic process, say salt retention or capillary permeability, and be mitigative without having any significant meaning with regard to the basic dysfunction or etiology.

To appreciate more fully the implications of the hypothesis of the serous inflammatory process, we must review the anatomy of the connective tissue We recall that the morphologic elements include cells, fibers and ground substance arranged in different proportions in the various "organs" of the connective tissue system-bone, cartilage, areolar, etc. Fibroblasts, mast cells, mesothelial or serosal lining cells and reticulo-endothelial cells represent some of the cellular components Collagen reticulum and elastin are the fibrillar structures The ground substance, though not always visible, is a jelly-like structure in which these other elements are embedded

Mere listing of the anatomic structures does not give us an idea as to

the reactivity of the tissue. We shall not discuss the functions of the vigorous reticulo-endothelial system, however, because of space limitations. Its role in degenerative diseases of the connective tissue has been discussed recently (Aegerter & Long, 1949). We will concern ourselves chiefly with certain of the chemical and physical properties of the ground substance particularly because we believe these characteristics are particularly germane to the pathogenesis of certain degenerative lesions.

The amorphous ground substance of the connective tissue is a jelly-like substance because it contains hydrophilic colloids. Chemically, these colloids are polysaccharides and they contain acid groups such as sulfate, carboxyl or acetyl. For these reasons, these substances are known as acid mucopolysaccharides (AMP) Hyaluronic acid, chondroitin sulfuric acid, heparin and mucoitin sulfuric acid are examples of this type of compound The jelly-like character of the material is related to the degree and asymmetry of polymerization When the compounds are depolymerized, their viscosity is destroyed and their jelly-like character is lost. (Meyer, 1947; Clark & Clark, 1933, Bensley, 1934).

In recent years, numerous researches (Chain & Duthie, 1940, McClean, 1930) have resulted in the identification of the "spreading factor" with hyaluronidase, an enzyme complex capable of depolymerizing and partially hydrolysing hyaluronic acid, one of the mucopolysaccharides of the ground substance. The action of the spreading factor, then, is believed to be due to the decrease in the viscosity of the ground substance and a lowering of this mechanical barrier. In spite of continued work on the subject, the significance of hyaluronidase in physiologic and pathologic and pharmacologic anti-hyaluronidases remains obscure (Glick & Gollan, 1948; Good & Glick, 1950)

Schade believed that the extracellular connective tissue components, the ground substance and fibers, were importantly involved not only in tissue permeation but also in water-electrolyte, acid-base and osmotic equilibria. The essential features of his view are that the two elements—the fibers and the ground substance-act as a two-colloid system whose individual properties are frequently antagonistic so that their "regulator" activity is enchanced Fig 2 illustrates some of the properties of these substances

In addition to these tabulated factors, specific ions are of some influence in determining the colloidal state of the connective tissue Increase in the concentration of the sodium ion, for example, is accompanied by

increased dispersion of the colloid and water retention whereas an accumulation of potassium or calcium is followed by shrinkage and loss of fluid.

The characteristically described movements of water and electrolytes in serous inflammatory lesions (Kaunitz, 1936) tend toward dispersion of the colloid and decreased viscosity, and, indeed, these latter findings have

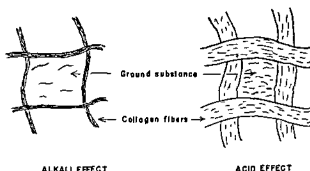


Fig 2

been described in rheumatoid arthritis (Ragan & Meyer, 1949). Since the reporting investigators did not describe associated electrolyte, ionic or pH changes, their conclusion that there is an abnormality in the formation of mucin is not entirely acceptable as yet.

The acid mucopolysaccharides when polymerized have the property of inducing metachromasia with certain basic aniline dyes. Metachromasia (meta, change + Gr. chroma, color), of course, refers to the property of staining a tissue element a color different from the original dye. Thus, toluidine blue, a blue dye, stains acid mucopolysaccharides a reddish-purple color. This "histochemical" test has been most useful.

In a survey of a number of pathologically occurring and experimentally induced lesions (Altshuler & Angevine, 1951), it was determined that the acid mucopolysaccharides or metachromatic substances were produced frequently in the subacute stages of the serous inflammatory lesions. As examples and to indicate the scope of the process, these substances are manifestly increased in lesions associated with rheumatic fever, rheumatoid arthritis, disseminated lupus erythematosus; in patients with diabetes, myxedema and pregnancy, after prolonged administration of estrogens, androgens, desoxycorticosterone and lyophilized anterior pituitary extract Clearly, the formation of these jelly-like substances cannot be correlated

with any particular extrinsic agent and the tissue itself must be examined to determine the pertinent factors.

In many, lesions associated with an increase in A M P's, an increase in mast cells may also be observed. This has led some investigators (Asboe-Hansen, 1950, 1952) to believe that the mast cells may actually produce the amorphous ground substance. This is somewhat difficult to visualize chemically and besides, it may very well be that those influences which encourage the production of acid mucopolysaccharides of the ground substance may also act similarly to produce the intracellular mucopolysaccharide of the mast cell.

To understand the significance and the nature of the tissue metabolic alterations associated with acid mucopolysaccharide formation is, of course, highly desirous and essential for real advancement in the problem of degenerative diseases For it is in this sphere that the promise of chemotherapeusis now seems most bright ACTH, cortisone and vitamin C studies, although far from the final answer, have given substance to this fanciful dream.

A study of the serous inflammatory lesions supplies us with many interesting leads in this regard It seems that the polysaccharides in question are most easily found in tissues which have a relatively poor vascular supply The metabolism of these tissues (designated bradyotrophic by some) is characterized by an increased glycolysis[1] (Dickens & Weil-Malherbe, 1936, Lipmann, 1942)

Bradyotrophic tissues (cartilage, synovia, renal medullary substrate, intestinal mucosa), as stated, are characterized by the ability to produce acid mucopolysaccharides Neoplastic (Dickens & Weil-Malherbe, 1936, Warburg, 1930; Lipmann, 1942), embryonal (Philips, 1941), partially anoxic and inflamed tissues (Kaunitz & Selzer, 1938; Fleckenstein, 1944) share this property of increased glycolysis and, interestingly, they too may contain increased quantities of acid mucopolysaccharides It seems, therefore, that given the necessary synthetic enzymes (fibroblasts, intestinal glands, e g.) and substrate, increased glycolysis may potentiate acid mucopolysaccharide formation.

Since lactic acid is formed in the glycolytic mechanism, it became of

[1] Glycolysis refers to the breakdown of carbohydrate with the formation of lactic acid Respiration refers to the complete oxidation of the carbohydrate through the state of pyruvic acid with the formation of the end products of carbon dioxide and water (Fig 3)

interest to determine whether this substance might not act as a building block for the polysaccharide. In an effort to determine whether this was true, radiolactate (Zn ($CH_3C^{14}HOH$ CO_2)$_2$ $3H_2O$) was repeatedly injected into the knee joint of a rabbit while serous inflammatory lesions were being induced Although production of AMP could be shown subsynovially in these animals, autoradiographs and direct counts on tissues

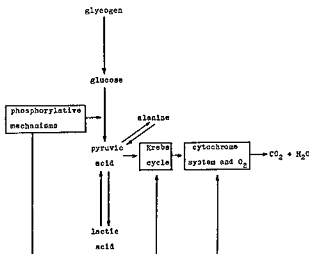


Fig 3

and synovial fluid failed to reveal any significant incorporation of the radioactive carbon. (Altshuler, 1953).

This finding may be considered to be in harmony with those of Topper and Lipton (1952) and of Roseman et al. (1952), who independently showed direct fixation of glucose – 1 – C^{14} into the acid mucopolysaccharide (hyaluronic acid) capsule of streptococcus hemolyticus. Whether these findings are applicable to humans, however, remains to be determined

Some interesting evidence is at hand concerning the glycolytic mechanisms in connective tissue. Lutwak-Mann (1940) has shown that it is difficult to get the usual fluoride effects in cartilage (possibly due to the presence of calcium or magnesium), that hexosediphosphate breakdown occurs at a pH greater than 7 4 a finding that may be pertinent because serous inflammatory lesions are associated with increase in tissue pH (Knepper, 1937, Koller et al, 1934); that methyl glyoxal may be a normal intermediate breakdown product of hexosediphosphate in the

formation of lactic acid, see also (Salem & Crook, 1950); that iodacetic acid, salicylates, bile salts and cincophen are without marked effect on the utilization of the phosphorylated sugar derivatives. These findings are provocative and suggest a possible variant of the usual glycolytic mechanism in connective tissue

The metabolic alterations of the tissue are undoubtedly conditioned significantly by concomitant electrolyte, nutritional and hormonal influences, and it is of some interest to recall that one electrolyte abnormality involves an increase in tissue sodium Apparently, then, the Na-inhibition of glycolysis reported by Utter (1950) in the brain preparations may not apply to connective tissue. Diets rich in sodium are said to potentiate the serous inflammatory lesions whereas low sodium diets are believed to ameliorate some of the changes

Similarly, abnormalities in vitamin B (Eppinger et al, 1935, Eppinger, 1949), pyridoxine (Rinehart & Greenberg, 1950), and vitamin C metabolism are reflected in changes of the connective tissue The work of Wolbach and collaborators (1926, 1933) indicated the importance of ascorbic acid in the metabolism of the intercellular material. Vitamin C was thought to be necessary for the production and maintenance of these substances It is of interest to note, however, that the action of ascorbic acid may be in the stage of conversion of the ground substance to collagen rather than in the formation of the metachromatic materials (Robertson, 1952). In scorbutic guinea pigs, for example, abundant chromatropic material may be seen in the lesions but collagenization is impaired (Altshuler & Angevine, 1951). However, contrary opinions have been expressed (Penney & Balfour, 1949).

Probably the hormonal effects on connective tissue have attracted the most attention The dramatic relief of clinical importance of ACTH and cortisone (Hench et al, 1949). Studies with these substances have indicated that they impair wound healing and the normal desmoplastic reaction to limit infections Here again much of the impairment of the normal connective tissue reaction is believed to be a matter of defective fibrillogenesis and/or the defective production of acid mucopolysaccharide (Taubenhaus & Amromin, 1949; Plotz et al, 1950; Spain et al, 1950). Desoxycorticosterone, thyroid, estrogens, and androgens are also of consequence

As stated, numerous studies dealing with the pathogenesis of the degenerative lesions of the connective tissue have indicated the basic similarities referred to above, and have stressed the importance of the ground

substance in the process. For example, Klinge (1933) and Bahrmann (1937) emphasized the role of the ground substance in fibrinoid formation, Aschoff (1921), Hueck (1938), Faber (1949) and others were concerned with the metachromatic substances in the pathogenesis of arteriosclerosis, Volland (1936) and Knežević (1944) related them to amyloid or paramyloid formation, and Loeschke (1927) and Leupold (1925) implicated the substances in hyalinization.

The exact mechanism by which these pathologic changes occur in the connective tissue is not known but the chemical properties of the acid mucopolysaccharides have suggested certain possible reactions, some of which follow:

(a) Protein reacting as a base + AMP → protein — AMP complex (Meyer et al, 1937)

(b) Complex protein + AMP → protein — AMP complex + liberated group, e g, cephalin-protein + AMP → protein + AMP complex + cephalin (Chargaff, 1938, 1942).

(c) Hydrophilic colloid + AMP → hydrophilic colloid + AMP
(dispersed) (flocculent)
(Cohen, 1942).

(d) (colloid) + (AMP) → (colloid — AMP) (Bungenberg de Jong,
(coacervate)
1936 and Koets, 1936).

(e) Carbohydrate + primary or secondary amine → nitrogen glycoside (Isbell, 1943) e g :

O=C–H, HCOH + N–H (ring) → N+ (ring) =HC, HCOH

To illustrate some of the problems involved in the attempts to determine the pathogenesis of the connective tissue lesions, we should like to review briefly some of the recent work on fibrinoid.

First, you will recall that the term fibrinoid refers to a homogeneous eosinophilic, refractile, relatively acellular band-like structure which has some of the tinctorial properties of fibrin The material occurs in many diseases affecting the connective tissue. It may be found in the so-called *diffuse collagen diseases,* including rheumatic fever, rheumatoid arthritis,

disseminated lupus erythematosus, periarteritis nodosa, in the bases of peptic ulcers; in placentae; in the vascular lesions of malignant hypertension It also may be seen following a number of non-specific damaging stimuli

It has been stated a number of times that fibrinoid is probably not an identical substance, in a chemical sense, in all lesions The age of the lesion, the anatomic site where it is formed and the method of damage probably all contribute to the exact composition of the material Continued experience, however, has indicated that the material generically is quite similar For that reason, it seems reasonable to apply a broad spectrum of observations in an attempt to determine its pathogenesis

The pathogenetic mechanisms that have been championed in the past include (a) necrosis of collagen (b) precipitation of the ground substance (c) exudation of fibrin or some other factor present in blood or (d) any combination or combinations of the above

The authors (Altshuler & Angevine, 1949) have expressed the opinion that fibrinoid forms by the co-precipitation of the AMP of the ground substance with a protein acting as a base

The arguments for this belief were.

(a) the temporal, spatial and configurational relationship of fibrinoid and metachromatic substances,
(b) the positive polysaccharide reactions of fibrinoid with periodic acid leukofuchsin,
(c) the increase in tissue pH and the liberation of alkaline substances in lesions generally associated with fibrinoid formation,
(d) the failure to obtain consistently positive fibrin (phosphotungstic acid hematoxylin), lipoidal or Feulgen reactions in the fibrinoid material, and
(e) the occurrence of fibrinoid or fibrinoid-like structures in areas where necrosis of muscle or of collagenic, reticular or elastic fibers cannot occur or where its occurrence is unlikely This opinion, it was stated, could only be conclusively established by the isolation and chemical analyses of fibrinoid material from various anatomic sites

Since that report, many other studies have been made available Using a "controlled" staining technique which is said to indicate the apparent isoelectric point of protein, Singer et al (1948) were unable to distinguish

between fibrin and fibrinoid. A similar study was reported by Sokoloff et al. (1951) who were also unable to differentiate the above materials from collagen on this basis The authors believed that these findings detracted from the argument that increased eosinophilia of the fibrinoid substances indicates the participation of a basic protein in its formation.

Other methods have been more successful in demonstrating a difference between these various structures. Kellgren & colleagues (1951), for example, showed in comparative histologic sections, electron micrographs and X-ray diffraction patterns that fibrinoid could readily be distinguished from either fibrin or collagen

Glynn & Loewi (1952) corroborated the older observation of Schlossmann (1942) that fibrinoid was resistant to tryptic and peptic digestion, whereas, fibrin is readily solubilized. They also distinguished fibrin from fibrinoid by the argyrophilic properties of the latter substance. Parenthetically it may be of interest to recall that certain in vitro acid mucopolysaccharide protein complexes are resistent to protein digestive enzymes.

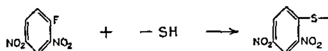
It is true, also, as stated by Dempsey (1950) that fibrin will give a positive reaction for aldehyde as determined by the periodic acid leukofuchsin technique Indeed, Consden & Stanier (1952) have been able to isolate carbohydrate from purified fibrin This reaction, therefore, should not be used to distinguish fibrin from fibrinoid and was not so intended by the authors in their study on the pathogenesis of fibrinoid.

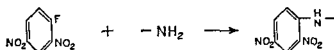
Further unpublished histochemical studies have been carried out by one of us (CHA) Since tryptophane and tyrosine are not present to any appreciable degree in collagen, it became of interest to determine the content of these amino acids in fibrinoid. Histochemical tests (Pearse, 1953; Glick, 1949—using the Voisenet-Furth and Millon reactions) for these substances were applied to tissue sections rich in fibrinoid material The reaction in fibrinoid is strongly positive and on these sections the degenerative material can readily be distinguished from the surrounding collagen These tests, again, will not distinguish fibrinoid from hyaline—like material occurring in blood clots.

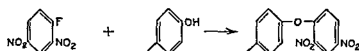
The sulfhydryl reactions using tetrazotized diorthoanisidine (Barnett & Seligman, 1952) and the reaction with 2,4 dinitrofluorobenzene (a modification of Danielli, 1950) were also positive.

The latter reagent is believed to react in the following manner:

It can readily be shown, as stated, that fibrinoid reacts strongly with this material and forms a highly colored product. This test was intended







to demonstrate the free amino groups in the protein but, unfortunately, the required preliminary destruction of the sulfhydryl groups with peroxide had not been carried out Thus, final interpretation of this result is not yet available

Certain microchemical studies have also been published. Consden & co-workers (1950) studied the composition of fibrinoid from freshly excised subcutaneous rheumatoid nodules These were separated as far as possible from surrounding connective tissue and then were washed, dried and defatted Collagen was removed by autoclaving with water (Lowry et al, 1941) and the residues and extracts were analyzed for hydroxyproline, tryptophane, tyrosine and reducing sugar Parallel analyses were carried out on normal subcutaneous connective tissue The results are as follows: (expressed as amino acid N as percentage total N).

Normal Connective Tissue	*Extract*	*Residue*
Hydroxyproline	8 2	1 1
Tryptophane	0 0	–
Tyrosine	0 7	2 5
Reducing sugar	1 3	–

Fibrinoid	*Extract*	*Residue*
Hydroxyproline	7 6	0 7
Tryptophane	0 0	0 8
Tyrosine	1 6	3 4
Reducing sugar	2 9	2 7

Before analyzing these data, it must be assumed that the "fibrinoid" examined is far from "pure" material A glance at the histologic section from a rheumatoid nodule indicates the difficulty in obtaining such a preparation by simple gross separation. Thus, the hydroxyproline present in the extract of the fibrinoid material may well indicate that collagen was present in the sample analyzed and not that it was a necessary component of the fibrinoid material. The same argument applies to the content of elastic tissue as indicated by the hydroxyproline of both residues Hydroxyproline, of course, is present in significant amounts only in collagen and elastic tissue

For purposes of calculation, the authors then assumed that fibrin was present in the fibrinoid material and showed that, even if it were, it would not account for the relatively great quantities of tyrosine present. It is to be remembered that (a) tryptophane and tyrosine is either totally absent or is present in small amounts in collagen and (b) the tryptophane to tyrosine ratio in fibrin is 4 : 7 (Haurowitz, 1950). Thus, even if all the tryptophane present would be assumed to have been derived from fibrin, the origin of much of the tyrosine would be unaccounted for.

Microscopic examinations and histochemical tests also indicate the difficulty of interpreting the meaning of the values found for the reducing sugar. Since abundant free polysaccharide is present in the rheumatoid nodule adjacent to and intermingled with the fibrinoid (Altshuler & Angevine, 1949), the finding of reducing sugar in the extract and residue needs correlative histologic data and better sampling techniques before it can be said to be an integral part of the fibrinoid material.

The work of Ziff, Kantor, Bien & Smith (1953) complements the above findings. In this study, fibrinoid was extracted from subcutaneous nodules by either dilute alkali or trypsin (Longer extraction periods,

TABLE 2[1]

Effect of	Ground substance	Collagen
Hydrogen ions	slight swelling	strong swelling
Hydroxyl ions	strong swelling	slight swelling
Distilled water	strong swelling	shrinkage and coagulation
Dilute salt solutions	strong swelling	slight swelling
Concentrated salt solutions	shrinkage	swelling

[1] Modified from Schade

mechanical shaking and difference in the state of the sample is said to account for the difference in the action of trypsin) The hydroxyproline content of the extract was then compared with the hydroxyproline content of the whole nodule. The authors also compared the relative areas of collagen and fibrinoid on projections of the microscopic sections From these projections, the approximate concentration of fibrinoid per gram of wet tissue was calculated. From the hydroxyproline content of the fibrinoid extracts, and the concentration of fibrinoid in the untreated tissue, the maximum percentage of collagen in the fibrinoid was calculated

From the data obtained in this manner, the authors conclude that if collagenous material is at all present in the fibrinoid of a rheumatoid nodule or in synovial membrane (Bien & Ziff, 1951) it was present in negligible quantities

Thus, though the final chapter has not been written, investigations have revealed some of the participating chemical elements and a clearer insight is being obtained in the formation of fibrinoid It appears that fibrinoid is distinct from both collagen and fibrin, and that the amorphous ground substance of the connective tissue probably participates in its formation It is definite that a protein is involved in its formation The alkaline nature of this protein, however, cannot be said to have been established with certainty, although no conclusive proof has negated that possibility

The above discussion, we know is only of heuristic value but it is our hope and belief that this approach to connective tissue lesions may aid in the planning of investigations and in the critical evaluation of clinical and experimental data

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CORTISONE, ASCORBIC ACID AND CHANGES IN THE RETICULO-ENDOTHELIAL SYSTEM

WITH SPECIAL REFERENCE TO THE PATHOGENESIS OF AMYLOIDOSIS

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Introduction

AFTER THE INTRODUCTION of cortisone and corticotrophin into the field of rheumatic and pararheumatic diseases it was found that these hormones would modify changes of connective tissue in natural diseases as well as in experimental conditions There is still another mesenchymal derivative which may play an important role in mesenchymal diseases, being involved in the synthesis of protein, the production of antibodies and the development of certain characteristic lesions, such as amyloidosis and hyalinosis This is the so-called reticulo-endothelial system comprising a specific tissue substance of the spleen, lymph nodes, bone marrow, liver, and other organs. Factors controlling structural and functional changes of reticulo-endothelial cells, and basic in the pathogenesis of amyloidosis and related conditions, will be the main subject for discussion.

NUCLEIC ACID AND SYNTHESIS OF PROTEINS AND ANTIBODIES

Systematic investigations carried out by Caspersson et al. and by Brachet have yielded important contributions to the question of the cytochemistry of the protein formation Brachet (1940) showed that the basophilia of various cells depended on the presence of ribose nucleic acid. Cells that were heavily stainable with pyronine lost this property after treatment with ribonuclease. The studies of Caspersson et al. (cf. bibliography 1950) have

led to the theory that the synthesis of protein is related to nucleic acid With special spectrographic and cytologic methods he and other investigators have obtained evidence from which it would appear that the multiplication of chromosomes is associated with the formation of desoxyribose nucleic acid, whereas the production of cytoplasmic protein is linked with that of ribose nucleic acid It is known that ribose nucleic acid occurs largely in cytoplasm, but also in nucleoli, whereas desoxyribose nucleic acid is restricted mainly to nuclei

Scandinavian workers, at first Bing & Plum (1937), Bjorneboe & Gormsen (1943), and Fagraeus (1948), demonstrated relationship between plasma cells and antibody production, and it may be assumed that the proliferation of cells of the plasmacytic type is associated with the production of antibody globulins Whether these are produced by immature or mature plasma cells, or by both, is still uncertain

Hyalinosis and Amyloidosis

Studies on hyalinosis, paramyloidosis and amyloidosis in mesenchymal diseases, including cases of probable hypersensitivity and associated with hyperglobulinaemia, showed an accumulation of plasma cells and other mesenchymal derivative cells showing a pyronine-positive material in cytoplasm during the active stage of disease (Teilum, 1948) Studies of sections from acute and subacute glomerulonephritis and paramyloid lesions in the kidney associated with hyperglobulinaemia showed, in addition to plasma-cell accumulations, pyroninophilia of proliferating cells of the glomeruli in the kidney, the adventitia of the vessels etc. Transitions from such cytoplasmic changes to prehyaline, hyaline or paramyloid substances were observed in a number of cases

Cases of probable hypersensitivity to sulphadiazine showing a widespread plasmacytosis and hyperglobulinaemia have later been reported by Robertson (1950) Klein & Block (1953) reviewed the relationship between marrow plasmacytosis, levels of plasma globulins, and the clinical conditions with which they are associated

Inhibitory Effect of Cortisone on Pyroninophilic Mesenchymal Cells

Adrenal cortical hormone of cortisone type has been shown to exert a pronounced inhibitory effect on plasma cells and other pyroninophilic cells

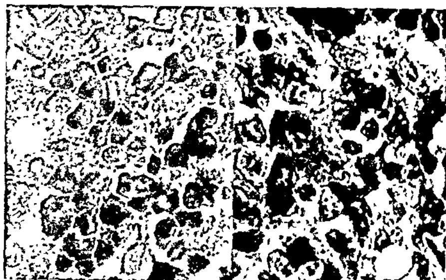


Fig. 1 a
Accumulations of plasma cells in the spleen of hyperimmunized rabbit

Fig 1 b.
Marked regression of plasma cells in the spleen of the same animal after injection of 20 mg cortisone acetate daily for 6 days Pyronine-methylgreen stain
(G Teilum, H C Engbæk & M Simonsen: Acta endocrinol. 5, 181, 1950).

in various organs Administration of cortisone (20 mg in daily hypodermic injections for 6 days) resulted in a widespread regression of the pronounced accumulation of plasma cells (Fig. 1 a, 1 b) in the spleen of rabbits immunized with a formaldehyde-killed Pfeiffer bacillus culture given in three weekly intravenous injections for several months (Teilum et al., 1950). In the animals treated with cortisone there was a marked rise in the α-globulin fraction and a less pronounced fall in the γ-globulin.

In rabbits hyperimmunized for periods varying from seven to 16 months the full picture of glomerulonephritis comparable with the natural disease (Ellis's type 1 and type 2 nephritis) was achieved in some cases. The proliferating cells of the glomeruli in acute glomerulonephritis (type 1) displayed a pronounced pyroninophilia which was abolished by treatment with ribonuclease The morphological lesions could be attributed to definite elementary cellular reactions controlled by adreno-cortical hormone. The effect of cortisone was observed in histological examinations of repeated biopsies from the kidney and the spleen. The administration of this hormone

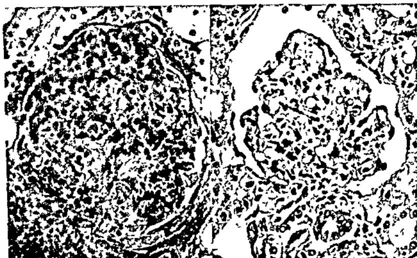


Fig 2 a

Proliferating cells (showing pyronine-positive material in cytoplasm) of glomeruli in acute experimental glomerulonephritis in rabbit (biopsy I)

Fig 2 b

Biopsy II Marked regression of pyroninophilic cells inside and outside the glomerulus and formation of homogeneous precipitate in the tufts after injections of cortisone acetate (G Teilum, H C Engbæk, N Harboe & M Simonsen J Clin Path 4, 301, 1951)

resulted in a marked regression of pyroninophilic cells inside and outside the glomeruli (Fig. 2 a, 2 b), and promoted a transition to homogeneous precipitates in the tufts, approaching the picture characteristic of type 2 nephritis (Teilum et al, 1951) The findings indicated that the adrenal cortex in some way conditions the response of the glomerular tufts in antigen-antibody reactions In cases comparable with type 2 nephritis further homogeneous material was precipitated in the tufts, whereas the number of cells decreased

The administration of cortisone in these experimental conditions resulted in a widespread inhibition of proliferating mesenchymal cells in the perifollicular zone of the spleen and in the glomeruli of the kidneys and the appearance of a hyaline or homogeneous amyloid-like substance

Pathogenesis of Amyloidosis

The varying results in staining reactions suggest that amyloid is not a uniform chemical substance, but a series of closely related protein compounds (Hass et al, 1943) Its composition may vary from one case to another and even in different areas within the same case. Amyloid is generally considered to be a compound of a globulin and a mucopolysaccharide In secondary amyloidosis, Hass (1942) identified two slightly different protein fractions and a sulphate-bearing polysaccharide, and showed that only 1–2 per cent of the amyloid substance is of carbohydrate nature Conditions in which secondary amyloidosis may develop have often been found associated with hyperglobulinaemia. Johansson & Wahlgren (1938) have shown that amyloid gives a distinct metachromasia with toluidine blue; this is considered a criterion of the presence of sulphuric acid ester of a high molecular weight, probably chondroitin-sulphuric acid.

Amyloidosis has been produced experimentally by various means, including repeated parenteral injections of bacteria, antigens, casein, nucleotides, human serum, sulphur, etc. The subcutaneous injection of nutrose (sodium caseinate) into mice, a method introduced by Kuczynski (1923), has been shown to be a most effective way of producing amyloidosis

Effects of Cortisone and Nitrogen Mustard in Inducing Amyloidosis

Experimental observations by Teilum (1951, 1952) have linked amyloid formation with a suppression of pyronine-positive mesenchymal cells which are in a protracted stage of active proliferation, the process being apparently controlled by the interaction of ascorbic acid and adrenal cortical hormone resembling cortisone

It was demonstrated that cortisone and corticotrophin exert a pronounced effect in inducing (or promoting) the deposition of amyloid In mice, treated over a period of weeks with injections of sodium caseinate, insufficient to cause formation of amyloid in the spleen (verified by histological examinations of biopsies), subcutaneous injections of 0 3 mg of cortisone daily for four to five days promptly resulted in the appearance of amyloid in the spleen (Fig 3 a, 3 b). Cortisone also induced amyloidosis when given simultaneously with caseinate, whereas the treatment with casein alone did not produce amyloid deposition within the same period. After injections of casein for a few weeks the spleen showed marked accumulations of pyroninophilic reticulo-endothelial cells, including many

plasma cells, when stained with Unna-Pappenheim's pyronine-methyl-green method

The pyroninophilia first appeared in the perifollicular zone of the spleen, whereas reticulo-endothelial cells of the liver and the glomeruli of the kidney were involved later and only very slightly.

Obviously, the amyloid deposition following depression of pyroninophilic cells by cortisone first occurred in the same sites as the preceding pyroninophilia

These findings cannot confirm the interpretation of amyloid as a process of infiltration or precipitation from the blood at the level of the reticulo-endothelial system, as it has been postulated by several workers

Since the principal systemic effect of the mustard compounds (a degeneration of the haemopoietic organs leading to involution of the spleen, lymphatic tissue, and the thymus) in many respects resembles the depressive effects of cortisone, the possibility was explored that nitrogen mustard, like cortisone, might promote amyloid formation under certain conditions. In mice, previously treated with injections of sodium caseinate for several weeks, three injections of nitrogen mustard (NH_2)—each equivalent to 2 5 to 5 mg per kg body weight—induced an almost diffuse amyloid deposition in the spleen (Teilum, 1954)

Altogether, there is evidence of inhibition of cellular proliferation in the reticulo-endothelial system to account for amyloid formation induced by various means (cortisone, nitrogen mustard)

The finding of pronounced pyroninophilia in the spleen and other organs, preceding the stage of amyloid formation, is in good conformity with the studies of the electrophoretic pattern in mice during the development of experimental amyloidosis Letterer (1949) thus found a higher γ-globulin increase in amyloidosis-affected mice than in non-affected mice after fifteen injections of nucleic acid Bohle et al (1950) found that the γ-globulin values in the serum rose sharply after twelve to twenty injections, whereas after thirty injections they were lower than the normal values.

√ Ascorbic Acid and Changes in the Reticulo-Endothelial System

The main effect of experimental ascorbic acid deficiency has long been known as an inability of the supporting tissue to produce and maintain intercellular substances. Collagen fibres are not formed, and the ground

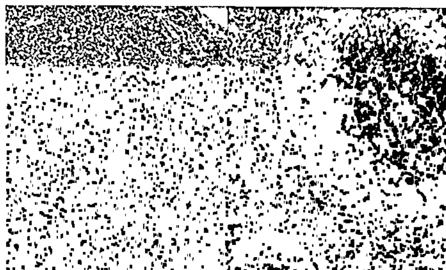


Fig 3 a Fig 3 b

Fig 3 a

Biopsy of spleen in mouse treated with casein injections for several weeks and showing no signs of amyloidosis

Fig 3 b

Marked amyloidosis in the same animal after further administration of 0 3 mg cortisone acetate daily for 4 days (Teilum, 1951, 1952)

substance is present in a depolymerized state (Gersh & Catchpole, 1949), leading to low viscosity and water solubility, which may account for the elevated serum levels of glycoprotein (Pirani & Catchpole, 1951) The connective tissue shows proliferation of a large number of mesenchymal cells which fail to mature *A priori,* it would be expected that also the reticulo-endothelial system was involved in the general dysfunction of mesenchymal tissue characteristic of vitamin C deficiency.

Pirani et al. (1949) observed marked amyloid deposition in guinea pigs fed on scorbutogenic diet for eight weeks or longer. Six out of seven animals of this group showed distinct amyloidosis, and in those showing amyloid deposition the spleen was severely involved, the liver moderately, and the adrenal cortex only very slightly involved in a few cases. According to these workers, their observations do not warrant any positive conclusion as to the possible role of ascorbic acid in the pathogenesis of amyloid deposition, but they point out that amyloidosis has not been produced previously in animals by means of a deficient diet.

Teilum et al. (1953) studied the changes in the reticulo-endothelial system in scorbutic guinea pigs, and found an initial proliferation of plasma cells in the spleen (Fig. 4), followed by reticulosis and deposition of an amyloid-like homogeneous substance in a later stage (Fig 5). At that time,

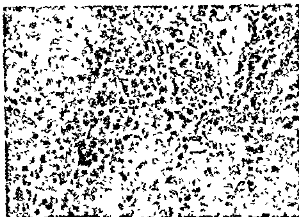


Fig 4

Initial proliferation of plasma cells in the spleen in scorbutic guinea pig

Pyronine-methylgreen stain

(G Teilum, N Harboe & H Lirck Acta path et microbiol scandinav 32, 109, 1953)

pyroninophilia had disappeared, and the lymphoid tissue showed atrophy with a marked reduction in the size of the malpighian bodies and the number of cellular elements. Prolonged administration of ascorbic acid caused a reappearance of plasma cells and a pronounced increase of the lymphoid tissue in the spleen, which was ascertained by biopsy. The electrophoretic studies of the serum proteins in animals with chronic scurvy showed a decrease of albumin and a marked rise in the α-globulin fraction. Principally, the electrophoretic pattern corresponded to that following the administration of cortisone to hyperimmunized animals

In hyperimmunized rabbits, too, showing regression of the pyroninophilia of the spleen, ascorbic acid induced a marked reappearance of pyroninophilic cells (Teilum, 1952)

Obviously, adrenal cortical hormone and ascorbic acid may influence the protein synthesis of reticulo-endothelial cells in a remarkable way. In most cases these effects are antagonistic Evidently, the cellular reaction of the reticulo-endothelial system in experimental ascorbic acid deficiency

comprises two phases: A primary stage of proliferation followed by a secondary stage of suppression of cellular elements—comparable with the reaction of the connective tissue in scorbutic animals.

The marked accumulation of plasma cells in the spleen and other

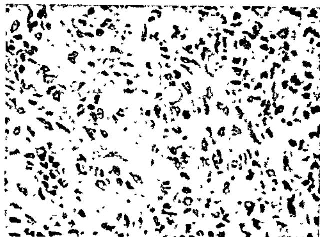


Fig 5

Deposition of PAS-positive homogeneous substance in spleen in chronic scurvy (G Teilum, N Harboe & H Lieck *Acta path et microbiol scandinav 32, 109, 1953*)

organs in the acute stage of scurvy (Teilum et al, 1953) seems to be of interest, not only in connection with the development of amyloidosis in this condition As the systemic intoxication induced in experimental scurvy resembles a pattern of organic changes in mesenchymal diseases and those observed under conditions related to stress, the possibility of a local ascorbic acid depletion as a basic factor in the genesis of mesenchymal and reticulo-endothelial reactions involved in a wide variety of disorders has been considered (Teilum, 1952). Fagraeus (1948) noted marked plasmacytosis in rabbits after injection of antigens, this effect being distinctly accentuated in animals which had been previously sensitized Long (1950) found that ascorbic acid influenced the secondary but not the primary immunity response. While the occurrence of plasmacytosis in infectious and allergic disorders may be considered as an immunity reaction, plasmacytosis of the haemopoietic organs has also been found in other conditions, e g. in atomic energy casualties (Liebow et al, 1949) and in chronic radium poisoning in rats (Thomas & Bruner, 1933). Dogs subjected to whole-body X-ray

irradiation regularly showed replacement of lymphocytes in lymphoid tissue by cells of a type that meets some or all of the morphologic criteria of the plasma cells (Wohlwill & Jetter, 1953).

Even though the problem remains essentially unsolved, the marked plasmacytosis in experimental vitamin C deficiency could explain the apparently non-specific nature of plasma cell response. According to this theory the similarity of essential alterations in stress phenomena in general and the changes in scurvy should be traced back to a common effect of ascorbic acid depletion as a response to a variety of stress conditions. In this connection, attention should also be paid to Dugal & Thérien's observations (1949) that, when large doses of [illegible] o guinea pigs, enlarge[illegible], although the ani[illegible] untreated controls.

Frequency and Significance of Amyloidosis in Rheumatic Disease

Whereas amyloidosis has been mainly considered a complication in chronic infective, tissue-destructive processes, such as tuberculosis, osteomyelitis and chronic bronchiectasis, a number of cases of rheumatoid arthritis and Still's disease associated with amyloidosis have been observed. Our own series (Teilum & Lindahl, 1954) showed an exceedingly high frequency of this complication, as 17 out of 28 cases (61 per cent) of rheumatoid arthritis (not treated with cortisone or corticotrophin) histologically showed amyloid lesions. Depositions of severe or moderately severe degree were present in 10 cases (35 per cent), whereas milder lesions were revealed in further 7 cases. Among the 17 patients with amyloid changes there were 13 who had albuminuria (76 per cent), and in all cases of albuminuria histologically varying degrees of amyloid deposition were found. Uraemia accounted for death in 25 per cent of all cases. It is worthy of note that amyloidosis had been diagnosed *clinically* in only 1 case, at autopsy in only 3 cases. An improvement of the joint symptoms occurred strikingly often at the time of onset of albuminuria, suggesting an inhibition of active phenomena in the disease.

Considering the high incidence of amyloid lesions in our series, the diagnosis of amyloidosis is justified in a patient who has had rheumatoid arthritis for a considerable period of time and who begins to excrete moderate to large amounts of albumin in the urine without evidence of other renal disease to account for the albuminuria.

Following prolonged cortisone treatment of rheumatoid arthritis which had set on 3 years earlier with pericarditis and acute rheumatism (in a 29-year-old man) West & Newns (1952) found development of an enormous amyloidosis in the liver, which at autopsy contained 95 per cent of amyloid substance and weighed 6470 gm Possibly a suppression of a marked fibroblastic proliferation of the liver associated with the pericarditis may account for the elective localization and degree of deposition in this case.

Generally, amyloidosis seems to be linked with a dysfunction of mesenchymal tissue. Its mucopolysaccharide component and metachromatic staining with toluidine blue indicates a near relation to the ground substance of connective tissue Metachromasia of the extracellular matrix is a characteristic alteration in the active phase of mesenchymal disorder, and, like the cellular changes, is controlled by adrenocortical hormone resembling cortisone and by ascorbic acid It is also known that the carbohydrate-ester, chondroitin sulphuric acid, is capable of combining firmly with several proteins to form stoechiometrically well-defined compounds (Meyer et al., 1937).

Various clinical observations, too, suggest a relationship between amyloid and collagen diseases. Lubarsch (1929) thus found amyloid nodes in the skin in clinical scleroderma. Transitional forms between scleroderma and primary amyloidosis are also known (Jørgensen, 1944). Stoeber (1934) described cases of allergic conditions combined with so-called genuine amyloidosis, and Cazal (1942) mentioned a case of amyloidosis in a 7-year-old girl with "un état anaphylactique" as the only aetiologic factor.

Amyloidosis in rheumatic disease differs from the ordinary secondary amyloidosis in that it represents in itself a typical morphologic phase of the mesenchymal disease. It is not another disease that sets on but a change in a mesenchymal, especially reticulo-endothelial, cellular function which has been involved in the disease already at an early stage.

The common essential factors involved in the pathogenesis of rheumatic disease, amyloidosis and stress conditions in general may account for the frequent and characteristic occurrence of amyloid lesions in rheumatoid arthritis, and may explain the amyloid deposition following treatment through a direct or indirect influence of the mechanism of adaptation.

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CONNECTIVE TISSUE AND INFECTION

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Microbiologists have studied extensively the part played by the bacteria and viruses in their invasion of the body and, particularly, how the various strains of bacteria and viruses differ in their virulence or ability to produce disease and why some can live free while others are dependent on the living cells of the host In regard to the host much has been learned concerning the manner in which the substances pass the epithelial barrier and the dissemination of infectious agents through the body by ways of the lymphatics and vascular systems. Until recently, however, very little was known about their spread through the connective tissue which is now understood to play an important part in the spread or localization of various disease agents.

The discovery of Duran-Reynals (1928) of the spreading factor and that of hyaluronic acid have led to the development of extensive studies in this field by scientists from a variety of disciplines. This work was excellently reviewed at a symposium conducted by the New York Academy of Science and published in 1950. The activity has resulted in a great extension of our knowledge of the connective tissue and a better appreciation of its importance, but it has also revealed that there are tremendous gaps in our knowledge which must be filled before we can have a satisfactory understanding of this important field.

A general discussion of the significance of the localization or spread of bacteria and viruses in the connective tissue of the host and the relationship of this to disease is of prime importance If in contact with the skin bacteria gain entrance through the epidermis to the dermis, the damage done by these bacteria will be greatly influenced by how rapidly or how slowly and over what area of tissue they disseminate themselves in a given period of time. By using bacteria of varying degrees of virulence Duran-Reynals (1935) was able to show that bacteria of low virulence when

concentrated in a small area of tissue cause a lesion, whereas if these bacteria are spread over a larger area, no lesion occurs Sprunt, expanding this work has found, but as yet not published, that the more virulent the organism is, the lower the concentration of bacteria per given area of tissue necessary to cause a lesion. In other words, there is a critical ratio of concentration of bacteria to tissue for each degree of virulence of the organism.

The picture of viruses, however, is somewhat different. Duran-Reynals (1935) in his early experiments showed that while he could suppress a bacterial lesion by spreading the bacteria over a large area, he was never able to suppress a virus by spreading it over a large area From this observation he reasoned that one unit of virus is sufficient to infect This conclusion is not absolutely correct, as Sprunt, Marx & Beard (1940) have shown that under controlled conditions it may take several hundred viral units to cause an infection It is true, however (Sprunt, 1939) that the greater dissemination of virus in the tissue, the greater the chance of a lesion. This decided difference between a bacterium and a virus is due to the fact that the bacteria live in the tissue spaces, whereas the viruses multiply in susceptible cells and are, thus, protected from the defense mechanisms of the host Therefore, the greater the area over which the virus is spread, the greater the opportunity of the virus reaching a susceptible cell Although there is no experimental proof, there is reason to believe that in regard to survival in susceptible cells the rickettsia and intracellular bacteria follow the rule of the viruses

An interesting analogy has been drawn by Sprunt (1950) between these bacteria and viruses going through the epidermis to the connective tissue of the dermis and a number of colonists landing on a strange shore If colonists land on an uncivilized and unfriendly shore, they can organize and through their united efforts build a stockade and possibly withstand the onslaught of the hostile natives Whereas, if they disperse over a large surface, and each individual or each small group of individuals tries to build a protection, they soon will be overcome by the natives and killed The topography of the land, on the other hand, might present numerous caves or other easily defended points, and the colonists could in widely spread groups of two or three hold out in one of these natural redoubts against the natives. The need for the bacteria to remain localized *is similar* to the situation of the colonists who had to remain together to protect themselves, whereas the viruses are similar to the colonists who dispersed over a large area but were able to defend themselves by entering caves,

susceptible cells in the case of the viruses. The action of the viruses and the bacteria may be called, therefore, the colonial effect.

The changes in the connective tissue which are influenced by the action of hyaluronidase on the polymers of hyaluronic acid, although far from being completely understood, are of primary importance. There may be changes of similar significance in the chondroitin sulphuric acid which is also part of the connective tissue matrix, but any consideration of this awaits additional work At the present a discussion of the changes due to the action of hyaluronidase on hyaluronic acid is all that available information warrants discussing Since the dramatic demonstration by Duran-Reynals (1928) of the effect of a small amount of testicular extract on the spread of India ink, it has been shown (Dorfman, 1950) that the viscosity of the polymers of hyaluronic acid in the connective tissue is greatly reduced by the action of the enzyme hyaluronidase and that this change in the viscosity is the result of a depolymerization of the hyaluronic acid.

The work of McMaster, which he and Parsons (1950) have summarized in the *Annals of the New York Academy of Science*, shows conclusively that there is no free fluid in the normal connective tissue. They proved this by demonstrating that there is no significant decrease in the spread of dye in the matrix of the dehydrated mouse as compared with the normal mouse Taylor & Sprunt (1943) supported this finding in the experiments in which they were unable to alter the spread of India ink in the skin of rabbits which were markedly dehydrated. They, as well as McMaster, were able, however, to decrease the spread of India ink or a dye in the skin by suitable hydration. Two methods, thus, have been demonstrated by which the viscosity of the connective tissue may be altered—the action of hyaluronidase on hyaluronic polymers and hydration of the animal These two factors are frequently interwoven as will be noted later.

Through the histochemical studies on the connective tissue matrix, which are extensively reviewed in McManus' article of this book, the numerous modifications of the viscosity of this tissue are clarified. The works of Bensley (1950) and others indicate that the connective tissue is altered with age and that this alteration probably explains in part the changes which occur with age in regard to infection. The interaction between the host tissue and the hyaluronidase in regard to a bacterium greatly facilitates an understanding of infections per se. Burnet (1948) has pointed out that digestive and respiratory mucins can be modified by

bacterial enzymes, this modification undoubtedly affecting the invasion of the bacteria

The significant part played by the connective tissue in infections is highlighted by the work of Lurie (1950) and Sprunt (1950). These authors have reported that changes in estrogen and gonadotropic hormones act respectively to decrease or increase the permeability of the connective tissue Sprunt (1950) first ascribed the effect of the estrogenic hormone by hydration of the tissue. However, as a result of the work of Duran-Reynals, Bunting & van Wagenen (1950) it seems more likely that the increase of water in the connective tissue under the influence of estrogen is not a simple hydration as all the water is bound The hormones produce hyaluronic acid polymers and, thus, cause the tissues to become more viscid As indicated by Catchpole, (1950) the gonadotropic hormones act on the polysaccharides of the connective tissue to make them more soluble, and, thus, the tissues become more viscid.

From the above findings it is seen that the viscosity of the connective tissue which determines the spread of infectious agents can be modified by the action of hyaluronidase either in the host or in the bacteria In addition to the hyaluronidase, there are perhaps other enzymes which hydrolyze or otherwise affect the various constituents of the connective tissue matrix

At present there are known (Duran-Reynals, 1950) to be other substances besides hyaluronidase which cause an increased spread of material through the skin, presumably by changing the viscosity of the connective tissue matrix but, according to present knowledge, without a hydrolyzing effect on any of the components Some of these substances are cevitamic acid, lecithin, and various peptones Because these have no effect on hyaluronic acid, Duran Reynals (1950) has suggested that they might act by altering another component of the matrix. So far, however, this theory has not been proved. Another possibility is that some of these substances activate bound hyaluronidase. The suggestion seems worthy of consideration A possible corollary of this is a dynamic equilibrium in many of the tissues between the amount of free hyaluronidase and the polymers of hyaluronic acid, this equilibrium being subject to alteration by factors which either increase or decrease the available free hyaluronidase. Modifications of this equilibrium, therefore, may be brought about by the substances mentioned above which release additional hyaluronidase and, thus, decrease the viscosity of the tissue Or, the reverse may be accomplished by such materials as antihistamine and salicylates which, as shown by Mayer

(1950), inhibit the action of hyaluronidase and, thus, increase the viscosity of the tissue. The action of the estrogenic hormone in causing an increase in the polymers of hyaluronic acid, therefore, is due to an inhibition of hyaluronidase.

Another important factor in the spread of infectious agents is the increased pressure caused by the diffusion of hyaluronidase through the connective tissue. Hechter (1950) demonstrated this influence experimentally by altering the pressure at the point of injection of the hyaluronidase. Changes in pressure in connective tissue occur *in vivo* as the result of localized edema and inflammation. As confirmed by Hechter, these changes act to increase the diffusion of the hyaluronidase present and, thus, modify the viscosity of the connective tissue with a consequent increase of the spread

In addition to the changes in pressure brought about by the release of hyaluronidase, Mayer (1950) has reported that inflammation affects the diffusion of material The exact mechanism is not understood, but Mayer & Kull (1947) consider it to be of particular importance in allergic reactions since it has been observed that antihistamine inhibits the action of hyaluronidase

In concluding from the above discoveries, one perceives that the extent of the spread of infectious agents through the connective tissue is decisive in the survival of these agents and, consequently, in the survival of the host This spread can be affected by a number of substances which alter the viscosity of the connective tissue. Up to the present these facts have not been extensively applied to disease. Lurie (1950), however, has observed that the severity of tuberculosis is increased in the experimental animals given gonadotropic hormones and decreased in animals receiving estrogenic hormones He believes this discrimination is due to the fact that the estrogenic hormones increase the viscosity of the connective tissue, whereas the gonadotropic hormones decrease it Therefore, when estrogen predominates, the tubercle bacilli are localized, whereas with gonadotropic hormones the reverse is true Lurie also states that one reason young women are more susceptible to tuberculosis is the fact that the organisms are localized during part of the menstrual cycle and then spread during the other part This alternation of condition is favorable to the disease because the organisms at first multiply when localized and then diffuse throughout the tissue.

It also seems feasible that the viscosity of the connective tissue in the

dermis may determine the development of acne. One may draw this conclusion since it has been shown that low grade organisms if concentrated in the dermis may multiply, whereas if allowed to spread, they may be killed off by the defensive factors of the host in the skin

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INFLUENCE OF HORMONES ON INFECTION

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Introduction

IT HAS LONG BEEN suspected that the endocrine glands might play an important rôle in the defence mechanism against infection. In the past, main attention has been given to the thyroid, adrenals and sexual glands, owing to the fact that these organs undergo a number of morphological changes during infectious diseases. However, several attempts made in order to obtain some protection by using impure extracts of these glands, both in human and experimental infections, failed to give conclusive results.

In the past few years, the adrenal cortical hormones attracted considerable attention after the discovery of Hench et al. (1949) concerning the effect of cortisone in rheumatoid arthritis A great deal of reports have appeared concerning the action of cortisone and corticotrophin on infections and many notable advances have been made in this revived field of research Our task is to analyse here the main facts which have been reported on this subject, in connection with our own research work; a short draft also will be added on recent work dealing with the pituitary somatotrophin

Effect of Cortisone on Infection

Early in the cortisone era, it has been reported that adrenal cortical hormones might be useful in the treatment of certain infections in man, as they possess an ameliorating effect on many signs and symptoms of the disease. Patients with viral and pneumococcal pneumonia, typhoid fever, Rocky Mountain spotted fever, peritonitis, viral hepatitis and scarlet fever, treated with cortisone, experienced a sense of well-being, an increase of the appetite, and a sharp drop in temperature. Considering this fact, hormonal treatment was recommended in certain infections, as a valuable adjunct of chemotherapeutic measures

At present, the favourable effect sometimes obtained by using adrenal cortical steroids in clinical practice is generally considered as due to some antitoxic activity of these compounds, similar to the one which may be observed in the treatment of exogenous or endogenous toxemias Actually, the improvement of the toxic symptoms is never associated with a corresponding improvement of the infectious state neither the course, nor the basic mechanism of the infection are substantially modified by the hormonal treatment

On the other hand, it should be pointed out that several clinical reports have been dealing with the appearance of infections in human beings treated with large doses of cortisone for collagen diseases, latent infections could be awakened by the hormonal treatment and their general spreading facilitated A great deal of work has been done on the clinical use of cortisone in human tuberculosis, but at present it is generally accepted that adrenal cortical steroids have no place in the treatment of active tuberculosis Actually, this therapy tends to lessen localisation of the disease and to facilitate widespread dissemination (Canada et al, 1952)

Experimental evidence seems to support the above mentioned statements Researches in this field have dealt with the occurrence of spontaneous infections in cortisone-treated animals and with the influence of this hormone on induced infections

It has been reported that in several animal species exceedingly large amounts of cortisone result in the frequent appearance of spontaneous and rapidly fatal infections In our studies the occurrence of such infections was very high, independently from the animal species and the type of experiment, provided that large amounts of hormone were given for a prolonged time in such instances, necrotic and ill-defined foci were found in lungs, liver and heart From the lesions several strains of organisms were cultured, which generally were found to have no ill effect when injected in animals without hormonal treatment These spontaneous infections, owing to their multiple and different etiology, were frequently found to be insensitive to antibiotic treatment, both penicillin and streptomycin, even at high dosage, did not lower consistently their incidence We have been able to confirm the view that there appears to exist a close relationship between the dosage of cortisone, the weight loss of the animal, and the development and spreading of infections It is remarkable that in the intact animal corticotrophin, even in large amounts, never caused the appearance of intercurrent infections (Cavallero & Sala, 1951, Cavallero, 1953 a).

Experimental studies concerning the effect of cortisone on induced infections have generally shown that this hormone alters the host's response to infectious agents. In a number of species, infected with many different organisms, this alteration has been deleterious to the host resistance Among the infections adversely affected by cortisone are those caused by pneumococci, streptococci, staphylococci, brucellae, typhoid bacilli, *Corynebacteria*, tubercle bacilli, poliomyelitis, influenza, mumps, encephalitis, vaccinia, Coxsackie and rabbit fibroma viruses, spirochaetes, trypanosomes, malaria parasites, *Trichinella spiralis* and several varieties of fungi As regards other cortical hormones, it has been shown that Compound F lowers the resistance to infections to the same degree as cortisone (Robinson & Smith, 1953), on the contrary, Compound B was observed to have a slight influence on some infections, but none in others (Kass et al, 1953). Adrenal cortical extracts and desoxycorticosterone appear to be inactive

In our laboratory some work has been done on the effect of cortisone on experimental mycotic infections A constant aggravation was observed in several types of mycosis and in different animal species Cortisone increased the morbidity and mortality rate and facilitated the spreading of the infection, the most striking results have been obtained when the animals were treated for a period of two or more days before the infection The deleterious effect has been evident by using both low and high virulent pathogens, when using apathogen strains, cortisone has never been found to be able to induce some change in the natural immunity of the host. Thus, cortisone lowers the resistance of the host against pathogens, but it is not able to sensitize the organism to apathogen germs (Cavallero, 1953 a)

It is important to note that the amounts of cortisone required to bring about lethal infections are extremely large; therefore, it has been suggested that the enhancing effect of the adrenal steroids is not physiological, but rather an unnatural pharmacological effect which bears no real relation to any physiological function of the adrenal cortex. Some workers have raised the interesting possibility that smaller doses of cortisone may have a quite different effect on infection, perhaps a protective one (Robinson & Smith, 1953) Others have suggested that cortisone might act indirectly on the host through its effect on the pituitary and the adrenal cortex, thereby inducing variations of other hormones which in turn might be the agents directly responsible for the effects observed (Shwartzman et al, 1950).

In connection with this hypothesis, we should like to summarize some

experiments we have made on adrenalectomized and stressed animals In these experiments, adult rats were infected intravenously with a fixed dose of *Monilia* Intact, adrenalectomized, and animals submitted to severe stress were used, cortisone treatment, 0.25 mg. daily per 100 gm. of body weight, was started two days before the infection and continued until the sacrifice, i e seven days after the infection. The pathological changes of the kidneys were evaluated according to the growth of the germ in the lesions and the extension, number and type of the infectious foci (Figs. 1–4).

TABLE 1

Pathology of the kidneys in intact, adrenalectomized (ADX) and stressed rats, infected with a low virulent strain of Monilia, effect of cortisone on the extension and type of the infectious foci and on the growth of fungi in the kidney lesions

Group	Control			Treated		
	Extension	Type	Fungi	Extension	Type	Fungi
Intact	+	exsudative	—	++	exsudative necrotic	+
ADX	+	exsudative	—	++++	necrotic	+++
Stress	++	exsudative necrotic	+	++++	necrotic	+++

It appears from the table that, in all groups studied, cortisone evidently aggravated the infection, this aggravation being most evident in adrenalectomized and stressed animals, in the latter groups hormonal treatment facilitated the germ growth and caused widespread necrosis From these results it appears that functional changes of the adrenal cortex may modify the effect of cortisone on infection, they give support to the suggestion of Ingle (1952) that the adrenal cortex might have some rôle as a conditioning factor of the cortisone effect

Effect of Corticotrophin on Infection

Corticotrophin has been largely investigated as regards its influence on both human and experimental infections Experimentally, it has been shown that generally there is little difference between the effects of corticotrophin and cortisone on a given disease but, in cases in which different actions have been observed, cortisone has appeared to be a more potent inhibitor of resistance than corticotrophin. Actually in certain infections, such as ex-



Fig 1. The kidney of an adrenalectomized, untreated rat, 7 days after the intravenous inoculation of *Monilia*, showing diffuse infiltration of lymphocytes and plasma cells; several giant cells are seen (Hematoxylin-eosin stain × 120) *Fig 2.* The kidney of an adrenalectomized and cortisone-treated rat, 7 days after the infection, widespread necrosis with scarce peripheral infiltration of lymphocytes (Hematoxylin-eosin stain × 120) *Fig. 3* Extensive necrosis in the kidney of an infected rat, submitted to severe stress and concomitantly treated with cortisone (Hematoxylin-eosin stain × 120) *Fig. 4* The kidney of a cortisone treated, adrenalectomized rat, showing proliferation of fungi in the necrotic foci (Gram-Weigert stain × 200).

perimental tuberculosis in mice, rats and guinea pigs (Le Maistre et al., 1953), type 2 pneumococcus and influenza A virus infections in mice (Kass et al., 1953) and tuberculous infection in some races of rabbits (Lurie et al., 1953), corticotrophin has failed to produce evidence of significant alterations. Thus, an important working hypothesis has been raised, i.e. the possibility that corticotrophin might liberate from the adrenals, at least in some animal species and in given conditions, some unknown substance to which the anti-infective activity of the gland might be ascribed.

Some observations we have made on pneumococcus infection in mice do not support this view. In intact mice, infected intravenously with type I pneumococcus, we have observed that both cortisone and corticotrophin (Armour), in small and high amounts, increased the mortality rate practically at the same degree; the combined treatment with cortisone and corticotrophin was additive, i.e. it resulted in a further increase of the mortality rate, while the contemporary administration of an aqueous cortical extract (Upjohn) did not counteract the adverse effect of cortisone. From these data we concluded that neither corticotrophin causes the adrenal cortex to secrete substances having a favourable influence on the course of infection, nor the adrenal cortical extract has a protective action (Cavallero et al., 1952).

Mechanism of the Cortisone Effect

Several mechanisms have been suggested, that may be involved in the action of cortisone on the process of infection. Cortisone might act upon the germ or upon the host. By direct action it might so affect the germ as to make it more virulent, but, in general, the germ virulence seems to be unaffected. Up to date, the evidence at hand suggests that the unfavourable effect of cortisone on infection is a direct result of some action on the host. In producing this effect cortisone may act by several ways, interfering with the humoral and/or the cellular defence mechanisms.

The first possibility is that cortisone might act on the antibody-forming process. Several data support the view that, under cortisone treatment, the release and/or the synthesis of antibody protein are inhibited, but at present it seems unlikely that cortisone enhances infection mainly or exclusively as a result of interference with formation or maintenance of adequate antibody. It seems more likely that this action is mainly due to changes in tissular immunity.

The main effects of cortisone on the early and subsequent steps of the inflammatory process are well known. Recent studies have emphasised that this hormone does not interfere with the early stages of the inflammation,

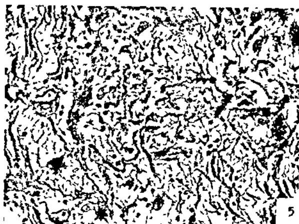




Fig 5 Control rabbit infected with myxoma virus, large accumulation of metachromatic ground substance at the site of inoculation (Hematoxylin-eosin stain × 200). *Fig* 6 Cortisone-treated rabbit infected with myxoma virus The intercellular ground substance is mainly represented by acidophilic material without metachromasia (Hematoxylin-eosin stain, × 200)

but only with the subsequent ones (Lattes et al, 1953; Lovell et al., 1953). The point that a reduction of capillary permeability may be caused by cortisone has been raised by several investigators; moreover, it has been claimed that cortisone could alter the polymorphonuclear leucocyte. The reticulo-endothelial system, which is considered to play the main rôle in

the defence mechanism against infection in general, seems to be particularly affected by cortisone. It has been shown that this hormone reduces the number of undifferentiated histiocytes, plasma cells and mast cells (Cavallero & Braccini, 1951, Cavallero & Pellegrini, 1951), concerning the clearing activity on bacteria of the reticulo-endothelial system, it has been recently concluded that the main effect of cortisone is to reduce the capacity of the system to remove, fix or detoxify bacteria or certain bacterial products (Thomas, 1953).

In our opinion, the effect of cortisone on the amorphous ground substance of the connective tissue must be kept in mind when studying the influence of this hormone on infection It has been claimed that the ground substance might be a barrier to the spreading of germs and perhaps an important factor for the start and completion of the inflammation In normal and pathological conditions it has been shown that cortisone decreases the amount of metachromatic material in connective tissue. Such an effect was clearly observed in some recent experiments we have carried out on infectious myxomatosis of the rabbit, a virus infection which causes strong accumulation of metachromatic substance at the site of inoculation and in other parts of the body In these experiments, cortisone treatment, 10 mg daily starting two days before virus inoculation, delayed or suppressed the signs and symptoms of illness, prolonged the survival time and resulted in an evident reduction of metachromatic material in the affected areas; withdrawal of cortisone determined a prompt appearance of the usual lesions (Figs. 5–6)

These data give further support to the above mentioned statement, besides, they suggest that the effect of cortisone on infections appears to be closely related to the pathologic changes caused by pathogens Where inflammation is not the prominent feature and accumulation of some particular material occurs, as in infectious myxomatosis of the rabbit, cortisone might obtain favourable results.

Effect of Somatotrophin on Infection

Recently, some reports have appeared dealing with the possible interference of pituitary somatotrophin in infectious processes, it has been claimed that this hormone might be able to counteract the adverse effect of cortisone on some infections (Selye, 1951, Lemonde et al, 1952; Du-

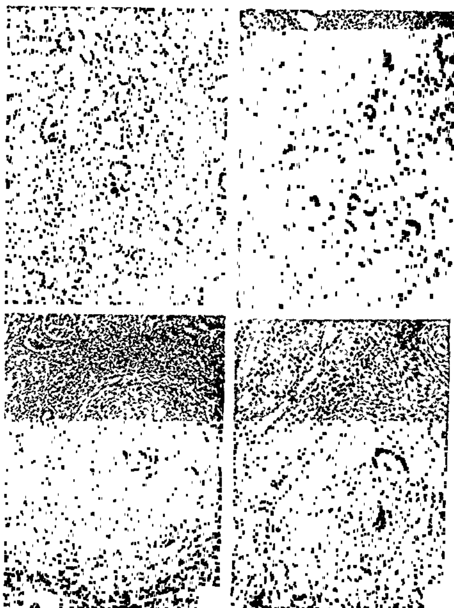


Fig 7 and 8 Diffuse infiltrative foci in the kidney of a control rat, 7 days after the intravenous inoculation of *Monilia*, mononuclear cells and giant cells are seen (Hematoxylin-eosin stain × 120) *Fig 9 and 10.* Granulomatous foci in the kidney of an infected rat, treated with pituitary somatotrophin, fibroblasts, large histiocytes and giant cells are numerous (Hematoxylin-eosin stain × 120)

commun & Ducommun, 1953; Horava & Selye, 1953; Pavlanis & Dufour, 1953). However, contrasting results have been reported too (Kass et al, 1953).

In immunological studies it has been observed that somatotrophin, in given conditions, might have effects opposite to those of cortisone (Hoene et al, 1953, Pellegrini & Andreoli, 1954). Morphologically, somatotrophin was shown to be capable of restoring the cortisone-suppressed fibroblast to normal and of stimulating proliferation of granulation tissue in intact and hypophysectomized animals, but not in adrenalectomized ones (Lichtwitz et al, 1951, Taubenhaus, 1953). In intact and hypophysectomized rats it causes strong accumulation of plasma cells in lymphatic organs (Cavallero, 1953 a, b) In our laboratory, previous experiments on rats with mycotic infections indicate that somatotrophin (Armour), 1 mg. daily per 100 gm. of body weight, exerts a stimulating effect on connective tissue, producing an excess proliferation of granulation tissue around the infectious foci (Figs 7–10).

It appears possible, therefore, that pituitary somatotrophin might have some rôle in the defence mechanism against infection, however, more information is needed before the question can be settled

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CONNECTIVE TISSUE AND CANCER

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Introduction

THE RELATIONSHIP between connective tissue and cancer gave rise to the first of the "modern" theories of cancerogenesis a century ago when Virchow postulated the transformation of the connective tissue cells associated with chronic inflammation into neoplastic epithelial elements. Although modified significantly by the later concepts of immutability of the germ layers, the basic connection between the chronic reactions to irritation and the subsequent development of neoplasms is as provocative today as when it was first suggested

Following the discovery of carcinogenic tars and hydrocarbons, research efforts were focussed on the search for new cancer producing chemicals and attempts to discover the direct biochemical and physiological effects of carcinogens on tissues and cells Interest in the interactions of tissues in carcinogenesis lagged for many years In the last ten years, however, many investigators have once again turned to this field in an effort to find common factors for explanation of the ever broader range of agents capable of evoking cancer

Two aspects of the relationship of connective tissues and cancer will be given our major consideration · first, the changes in connective tissues in which neoplasia occurs, and second, the connective tissue changes associated with precancer and cancer in associated epithelial tissues

Tumours of Mesenchymal Tissues

ORIGIN AND DIVERSITY

Spontaneous mesenchymal tumours of man, including all of the varieties of lymphomas, leukemias, and sarcomas as well as their many benign

counterparts, display cellular elements related to almost every type known to occur in normal connective tissues. Although histologically similar tumours either occur spontaneously or can be produced by carcinogenic agents in experimental animals, their specific cellular origins are as yet unknown

Connective tissues respond by neoplasia to a wide variety of agents, including all of the common carcinogenic chemicals, the carcinogenic viruses, certain parasitic infections and several types of penetrating radiation. In many cases the response may be controlled by proper choice of carcinogen and by its application in relatively high concentration to the site selected The living carcinogenic agents, such as the Rous virus, exhibit a high degree of tissue specificity, other agents, diverse as they are, appear to be quite nonspecific in their sites of action and provide no evidence that the fundamental mechanisms of neoplasia differ from tissue to tissue, nor from one kind of carcinogen to another.

Ground Substances and Mast Cells in Mesenchymal Tumours

The difference between carcinomas and sarcomas in their effects on stromal mast cells would appear to be an important clue to the study of these types of neoplasms The mast cell response to precancer in epithelia *and the absence of such cells from the stroma of formed carcinomas will* be discussed later Here we may consider the possible significance of mast cells and free stainable polysaccharides in the stroma of mesenchymal neoplasms.

An extensive survey of the occurrence of mast cells in tumours of mesenchymal origin was reported by Sylvén (1945). In association with the rapidly proliferating portions of most mesenchymal tumours, Sylvén demonstrated the presence of free mucopolysaccharides (stainable by Lison's method) and numerous mast cells The distribution and varying granular content of the mast cells were interpreted by Sylvén as evidence that they were responsible for transporting high molecular ester sulfuric acids to the growing portion of the malignancy. Such a view was consistent with his earlier interpretation of the role of these cells in wound healing and other reparative processes (*Sylvén, 1941*).

Experimentally induced rat sarcomas generally contain numerous, though irregularly distributed, mast cells (Holmgren & Wohlfart, 1947). These authors suggested that mast cells are probably formed locally from

other types of connective tissue cells and that they take part in the reaction of the system against tumour cells

Regulation of the physical state of the ground substance of the stroma of a transplantable sarcoma was ascribed to the activities of fibroblasts by Gersh & Catchpole (1949). It was assumed that some fibroblasts of rapidly growing tumours liberated "enzymes" that depolymerized the ground substance and made it more fluid Other fibroblasts with an elevated content of stainable glycoprotein presumably secreted this glycoprotein in an "effort to reverse this change in the physical state of the ground substance of the stroma" Although Gersh & Catchpole used the improved histochemical method for demonstrating polysaccharides as described by Hotchkiss (1948), they did not employ classical staining methods for demonstration of mast cells Their description of "fibroblasts" containing "glycoprotein" granules stainable with periodic acid-Schiff's reagent leave uncertain the kind of cells they actually observed

That there is an association between stainable mucopolysaccharides, mast cells, and rapidly growing mesenchymal tumours seems to be unequivocally established. More is known about the nature of these mucopolysaccharide substances than when Sylvén (1945) studied them Yet we are in the unenviable position he found himself in of passing off this association by saying that those substances "may have some unknown biological function to perform in connection with normal, as well as tumorous growth processes".

Relations between Connective Tissues and Carcinomas

The Desmoplastic Reaction

The development of cancer in an epithelial organ is associated with concurrent alterations in the supporting tissues of that organ Connective tissues provide the stroma and vascular supply for the carcinoma. Fibroblastic proliferation induced in adjacent connective tissue by a carcinoma, or desmoplasia, may be associated with the degree of host resistance to the invasive epithelial neoplasm. Mammary carcinoma explants have been shown to stimulate increased growth of fibroblasts in tissue culture (Ludford & Barlow, 1944), and when placed in the anterior chamber of the eye in contact with a bit of lung tissue from a newborn mouse, call forth proliferation of undifferentiated mesenchymal cells from the lung (Brow-

ning, 1952). The nature of the material that induces such proliferation of connective tissue cells is unknown.

Control of the stromal reaction to heterologous transplants by cortisone alone or in combination with total body x-radiation has been reported by Toolan (1953). Such techniques make possible the transplantation of human malignant tumours to rats and hamsters as well as reciprocal transplantations of these tumours between the latter species Although their usefulness is still to be tested, permanently transplantable human tumours in laboratory animals now appear to be more than a possibility.

Beyond simple description, we know little about the desmoplastic reaction to carcinomas. The importance of understanding host reactions to cancer, and especially the mechanisms by which the host attempts to combat cancer, is so obvious as to require no qualification In this area is one of today's richest cancer research opportunities.

Connective Tissue Reactions in Experimental Epithelial Carcinogenesis

The availability of a number of diverse chemical carcinogenic agents has made possible the study of experimentally produced cancer in almost every organ of the body While attention has most often been directed toward understanding the nature of the changes induced in epithelial tissues, it has been recognised that the effects of carcinogenic chemicals are by no means limited to epithelia Alterations in the supporting connective tissues accompany and are probably an essential part of the carcinogenic process in every organ subjected to chemical carcinogenesis. Inasmuch as skin has been subjected to more intensive study than most other organs, our consideration will be limited to this organ

Highly active carcinogens, such as methylcholanthrene, dissolved in benzene or acetone inflict severe damage on all epithelial and connective tissue elements of the mouse skin when first applied to the surface by painting Cramer & Stowell (1942) likened the effects of methylcholanthrene on the dermis to those induced by the severe trauma of a deep burn, noting that in both cases dermal fibers were disrupted and a variety of wandering cells, including macrophages, was to be found in the dermis Reorganization of the dermis was more orderly following a burn than after the "trauma" induced with methylcholanthrene. Repeated contacts with the carcinogen leads to the development of cells in both the epidermis and the dermis that are apparently relatively resistant to the destructive effects

of the carcinogen (Simpson & Cramer, 1945; Howes, 1946; Schober, 1951). Ma (1949) reported that collagenous and elastic fibres were increased in number in early stages of methylcholanthrene induced skin carcinogenesis in mice and later decreased These morphological studies were confirmed by chemical determinations of the amounts of collagen and elastin extractable from such skins.

Skin Grafting and Epidermal Carcinogenesis

Further evidence on the role of dermal changes in epidermal carcinogenesis has been obtained from recent studies of transplantation of skin of mice subjected to the local action of a carcinogenic hydrocarbon Novel techniques for grafting different skin components were devised by Billingham & Medawar (1951) and subsequently used by Billingham, Orr & Woodhouse (1951) and Marchant & Orr (1953) in an attempt to analyze the respective roles of the epidermis and deeper skin tissues in chemically induced carcinogenesis. The authors demonstrated that the carcinogen methylcholanthrene in acetone induced no persistent neoplastic change in the superficial epidermis that allowed it to progress to the development of a carcinoma when grafted to an untreated site in the same mouse Conversely, carcinomas often developed at the site of previous application of methylcholanthrene when the denuded area was covered by a thin graft of epidermis from the tail or some other part of the body Technical limitations prevented the complete separation of all epithelial elements from the dermal connective tissues, however, and a conclusive evaluation of the role of the dermis in development of epidermal cancer could not be made With their interpretation that the dermis plays an important role in the induction of neoplastic changes in the overlying epidermis we are in agreement; but believe more critical tests must be devised before this can be considered unequivocally proved

Mast Cells and Epidermal Carcinogenesis

The relationship of mast cells to experimental cancer and precancer is so striking as to deserve special attention The accumulation of these cells in the skin of animals subjected to the application of carcinogenic tar had been recognized for many years Massive local accumulations of mast cells in association with methylcholanthrene induced precancerous hyperplasia

of mouse skin were reported by Cramer & Simpson (1944), who associated the presence of these cells with resistance to epidermal neoplasia The same report included observations that mast cells disappeared promptly from the stroma of an invasive carcinoma when it arose from such precancerous skin, in contrast to their persistence in the stroma of newly formed sarcomas.

Comparable differences exist between human carcinomas and sarcomas in their relationship to mast cells. Earlier we have noted Sylvén's (1945) observations on the frequent occurrence of mast cells in the stroma of rapidly growing mesenchymal tumours In contrast, few if any mast cells were found by Janes & McDonald (1948) in the stroma of carcinomas of prostate, stomach, duodenum, breast, rectum, sigmoid, ureter or kidney, and none in the lymph nodes involved by metastatic growths. Many mast cells were found in nodes draining organs containing carcinomas as long as neoplastic cells had not reached the nodes.

Sylvén (1941) considered the presence of mast cells with few granules in healing wounds as evidence that these cells had given up chromotrope substance to the ground substance. We cannot here review the long standing controversy on the nature of this metachromatic substance. Many workers have published on this subject, the two most popular opinions holding that mast cells contain heparin or hyaluronic acid. Asboe-Hansen (1950, 1952) recognized that the metachromatically stainable granules of mast cells are not chemically identical with either heparin or hyaluronic acid and considered that they were probably undefined precursors of the hyaluronic acid of the ground substance. The disruption and vacuolisation of these cells, with loss of granular stainable material, following the administration of ACTH or cortisone has been interpreted (Asboe-Hansen, 1950, 1952; Cavallero & Braccini, 1951; Stuart, 1951) as evidence that the mast cells are end organs in the mediation of the effects of these hormones Continued cortisone treatment of mice reduces the number of mast cells, and also interferes slightly with the development of papillomas in response to 9 10 dimethyl 1.2 benzanthracene (Engelbreth-Holm & Asboe-Hansen, 1953) The mechanism of this effect is still obscure

The source of the tissue mast cells in precancerous skin has not been established with certainty, nor has the physiological significance of the variants of these cells been determined. One opinion holds that the mast cells are formed from other connective tissue cells locally. In this view the small cells containing few granules represent early stages in cytogenesis of

mast cells This concept is strengthened by the observation that young actively growing mast cells in tissue culture are found to have a small content of granules and that with maturation the granule content increases (Zitcer, Elsasser & Kirk, 1953). These superficial cells in hyperplastic mouse skin contain a labile chromatrope substance, as demonstrated by their lack of metachromatic staining by toluidine blue following aqueous formol fixation (Cramer & Simpson, 1944). Extraction of the chromatrope substance is not accompanied by the swelling and vacuolisation of the granules described for normal peritoneal mast cells of the mouse when subjected to distilled water or saline (Zollinger, 1950) and must be attributed to a lack of firm binding of chromatrope substance to the mast cell granules

A golden-brown fluorescence of these "ametachromatic" mast cells in frozen sections following aqueous formol fixation has been described (Cramer & Simpson, 1944; Simpson & Cramer, 1945) Figure 1 illustrates such fluorescent mast cells in a frozen section of mouse skin rendered hyperplastic by the action of methylcholanthrene in benzene In figure 2, which is of the same section after staining, the mast cells are seen coloured with toluidine blue. Many such comparisons leave no doubt that the golden-brown fluorescence is actually from mast cells and not from "macrophages loaded with fluorescent substances" as was suggested by Holmgren & Wohlfart (1947)

The larger cells full of metachromatic granules seen midway in the dermis, may be looked upon as differentiated or adult cells, moving downward as they mature. Deep in the dermis are found the largest and most variable mast cells, many of which show evidence of degeneration and disintegration. Staining variations and associations between "degenerating" mast cells and iron containing pigments are common in such areas These observations (Simpson, unpublished data) seem to confirm the reports of Sabrazès & Lafon, quoted by Deringer & Dunn (1947) as well as the findings of the latter investigators Many of these cells appear to be degenerating mast cells, but the possibility cannot be ruled out that they are macrophages containing, in some cases, phagocytized mast cell granules. Of interest but undetermined significance is the fact that such cells fluoresce intensely yellow-white in ultraviolet light This fluorescence is strikingly different from that previously described in the superficial "ametachromatic" mast cells

The foregoing interpretation of the local origin of mast cells is not

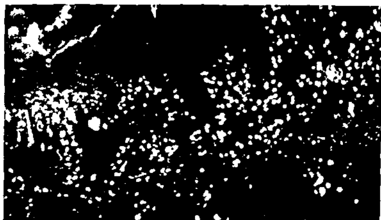


Fig 1

Photomicrograph of autofluorescence from unstained frozen section of mouse skin in which precancerous changes were induced by the application of 0 6 % methylcholanthrene in benzene White spots represent fluorescence from mast cells in the dermis Magnification 190 X.



Fig 2.

Same section as in Fig 1 after staining with toluidine blue for mast cells Comparison with Fig 1 shows that the majority of the stained mast cells here are identical with the cells in Fig 1 from which intense (golden-brown) fluorescence has been recorded. Magnification 190 X.

accepted by others, who hold that they come either from the blood or by migration from nearby tissues (Holmgren & Wohlfart, 1947). Sylvén (1941, 1945), Asboe-Hansen (1950), and others have looked upon dissolution of mast cells with release of metachromatic material as evidence that these cells carry materials to the ground substance.

In contrast to both of these views is the recent critical opinion of Devitt et al. (1954), who contend that the variability of the mast cells in normal tissues is as great as that described by others as the result of diverse physiological manipulations Workers who have actively investigated these cells in various pathological states will find it difficult to agree with the conclusion that the "morphologic features of the tissue mast cell are too variable and artifacts too frequent to allow any valid interpretations".

The growth of our knowledge of tissue mast cells and ground substance in recent years is most exciting Considered by many workers as biological curiosities until recently, mast cells have now been shown to display remarkable morphological and histochemical variations, especially in connection with the precancerous state of skin. Their further study in this condition and in other states in which alterations of the tissue mucopolysaccharides occur should be encouraged as a promising approach to the understanding of tissue permeability and possibly physiological mechanisms for the regulation of growth.

Invasion of Connective Tissues by Carcinomas

Still unfinished is the exciting chapter of cancer research on the subject of invasion and metastasis With the discovery of mucolytic enzymes in cancer tissue and the knowledge that appropriate substrate materials were normally present in the ground substance of connective tissue, it once appeared that the vital question of invasive growth was nearing solution Preliminary studies of the direct effects of injection of hyaluronidase adjacent to transplantable squamous carcinomas of the mouse resulted in the enhancement of invasiveness and metastasis by the tumour (Simpson and Gopal-Ayengar, 1947, Simpson, 1950) Contradictory results were obtained on transplantable sarcomas by others (Coman et al, 1947), and our subsequent experiments failed to confirm the effect of hyaluronidase on carcinomatous invasion when other transplantable tumours were studied (Simpson, 1950) Further doubt was thrown on the concept when Kiriluk, Kremen & Glick (1950) reinvestigated the hyaluronidase content of a variety of human cancers and found the enzyme present only when bacterial contamination was also present *Of as yet undetermined significance* is the fact that a high level of hyaluronidase inhibitor appears in the blood of patients with disseminated cancer (Kiriluk, et al, 1950) and with certain leukemias and lymphomas (Henstell & Freedman, 1951). The latter

investigators found a parallel between the effect of cortisone or ACTH on the serum antihyaluronidase titre and the clinical condition of the patient. Decreased titres following treatment were associated with corresponding clinical improvement



Fig 3

Section of mouse skin cut through site of intradermal injection of .01 ml hyaluronic acid solution (26 mg per ml) twelve days before animal was sacrified. Section shows intense cellular infiltration in dermis, destruction of muscle and massive hyperplasia of epidermis with loss of hair follicles and sebaceous glands Haematoxylin and eosin stain Magnification 52 X.

Although hyaluronidase production by carcinomas seemingly cannot explain invasive growth, the hypothesis is still attractive that some sort of mucolytic enzyme, as yet unidentified, is probably secreted by cancer cells to open the way for their invasion into adjacent tissues. Collagenase has also been considered as such a mechanism, but was found to be inconsistently associated with a variety of induced rat hepatomas and human tumours (Gersh & Catchpole, 1949). Coman (1953) discounts the possibility that mucinases are present in cancer tissue and looks to amoeboid movement and decreased adhesiveness of cancer cells for an explanation of invasiveness.

Effects on Epithelium of High Local Concentrations of Hyaluronic Acid

In earlier parts of this chapter the effects of carcinogenic chemicals on the skin have been reviewed and compared with the effects of a severe

localised burn trauma. Evidence has accumulated that the dermis participates in some essential way in the process of epidermal carcinogenesis, suggesting the need for more intensive efforts to understand how the con-



Fig 4

From same mouse as Fig 3 Toluidine blue stained section shows presence of mast cells in dermis and occurrence of very numerous mitotic figures in basement layer of epidermis and in pegs of epithelium presumably derived from hair follicles
Magnification 290 X

nective tissues and epidermis may be functionally related in the development of cancer

Several years ago the author speculated on a type of interaction of epithelium and the mucopolysaccharides of adjacent connective tissues that might explain the development of invasive capacities of carcinomas (Simpson, 1950) On the assumption that mechanisms for production of mucolytic enzymes are normally present in embryonic types of epithelia and lost or inactivated in the course of differentiation, the growth of adult type epithelia in the presence of an abnormal type or quantity of mucopolysaccharide might favor, either by adaptation or selection, the development

of cells in which the production of mucolytic enzyme is reactivated. Several direct studies of the effects of high local concentrations of hyaluronic acid have been made in the author's laboratory.

A brief abstract of studies on the effects of human umbilical cord hyaluronic acid (generously donated by the Schering Corporation, Bloomfield, N. J.) on the dermis of mouse skin and on the growth of epithelial

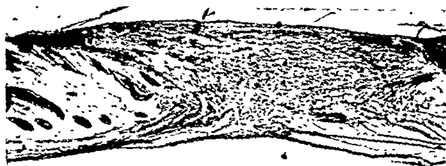


Fig 5

Mouse skin nineteen days after intradermal injection of .01 ml viscous solution of hyaluronic acid Heiderhain's modification of Mallory's connective tissue stain shows accumulation of collagenous fibers in dermis and loss of normal epidermal appendages Area resembles skin with a burn scar at this time. Magnification 52 X.

cells in tissue culture has been published (*Shifrin, Keller & Simpson*, 1950). Although inhibition of growth of embryonic chick iris epithelium was the only effect consistently observed in tissue culture, the effects of the intradermal injection of a minute quantity of a viscous hyaluronic acid solution into mouse skin were extensive and striking (Simpson, unpublished observation). Certain significant features of this reaction are illustrated in figures 3 to 5. In general, the early effects of hyaluronic acid on the dermis are those of acute inflammation. This is followed by regeneration of a highly cellular dermis and a concurrent intensive hyperplastic reaction on the part of the epithelium Early stages of this reaction, up to approximately 2 weeks, resemble faithfully the early reaction to a carcinogenic hydrocarbon After about 2 weeks the reaction subsides and leaves by the third week a scar with all of the characteristics of that produced by a severe localised burn. It is believed that the changes observed are rather specific for hyaluronic acid, for much less effect was observed following the intradermal injections of other polysaccharides and other viscous solutions (agar,

dextran, starch, gelatin). The lack of a vigorous stromal reaction to implants of agar was also noted in the control animals studied by Koche (1953). The significance of the reaction of skin to hyaluronic acid must be assessed after further study, but it obviously suggests a possible common mechanism for the expression of the effects of severe trauma and of carcinogenic agents.

Conclusion

It is hoped that this limited review may serve to show that cancer, at least when experimentally induced, is not the simple response of an individual type of cell of any single tissue. Interactions between cells and tissues, both locally and in remote parts of the organism are regular parts of the carcinogenic process and cannot be assumed to be incidental. Investigations of the relations of connective tissues to the development of cancer have now moved to the forefront of basic research Continued and expanded efforts in this area should be encouraged in the hope of bringing about an early understanding of the basic mechanisms by which neoplasia occurs, and through this effective measures for its control

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OBSERVATIONS ON THE HISTOGENESIS AND PATHOGENESIS OF ARTERIOSCLEROSIS

WITH NOTES ON EXPERIMENTAL ARTERIOSCLEROSIS OF PYRIDOXINE DEFICIENCY

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Introduction

RECOGNITION OF GROUND SUBSTANCES as important constituents of connective tissues is a relatively recent development Substances of this character are widely distributed, and an increasing realisation of their importance in health and disease has stimulated considerable research in a wide range of biology (*Ann N. Y Academy of Sciences*, 1950)

Studies which we have conducted indicate that a 'ground substance' is not only an important constituent of normal blood vessels, but that alterations of this material constitute a major feature in the evolution of arteriosclerosis (Rinehart & Greenberg, 1949, 1951)

Our interest in the mucinous component of blood vessels was stimulated by study of arterial lesions which developed in monkeys subjected to protracted pyridoxine (vitamine B_6) deficiency It was soon apparent that the lesions, which are remarkably like those of human arteriosclerosis, were typified either by a swelling of pre-existing mucinous ground substance or by deposition of increased amounts of such material in the walls of arteries.

This investigation has been supported by the National Heart Institute of the United States Public Health Service and the American Cancer Society

Material and Methods

Illustrative material relative to this report is derived from experimental and human material. The experimental material is naturally somewhat better for illustration because of prompt and controlled fixation.

Until recently, demonstration of ground substance of various connective tissues including blood vessels has been dependent upon its metachromatic staining reaction with toluidine blue of thionine (Bunting, 1950). While this reaction has been of great value in the study of ground substances, it is subject to certain vagaries which are dependent upon the stain, fixation and other technical variables and does not clearly delineate the finely distributed ground substance. In addition to staining with toluidine blue, three relatively new histochemical methods which are useful in the study of connective tissues have been employed in this study. One, based on the Hale (1946) technique, is dependent upon the affinity of acid mucopolysaccharides for colloidal iron and their subsequent delineation by the Prussian blue reaction with ferrocyanide. In the modification developed by us (Rinehart & Abul-Haj, 1951 a) which includes appropriate counterstaining with cochineal and fuchsin, the ground substance stains *blue*, 'basement membranes' stain *yellow* to *orange* and collagen fibres stain *red*. Elastic fibres are *unstained*, and as such are clearly outlined A combination of the colloidal iron technique and the periodic acid-Schiff reaction has also proved useful in our studies. With this technique 'basement membranes' stain a crisp red, collagen also stains red, but less sharply. A modification of Gomori's (1950) aldehyde fuchsin staining, which we believe selectively stains the sulphated acid mucopolysaccharides (Rinehart & Abul-Haj, 1952), has proved particularly useful in the study of blood vessels With this staining procedure the sulphated mucopolysaccharides stain violet, and the most delicate elastic tissue fibres are very sharply outlined There is certainly an intimate relationship between the sulphated mucopolysaccharide and the elastic tissue fibres in blood vessels Elastic fibres are bathed in a mucinous ground substance. It is possible that the elastic fibre proper is not stained by the aldehyde fuchsin but is outlined by condensation of sulphated mucopolysaccharide at its surface. Study of many blood vessels stained by this and other technique strongly suggests that the elastic tissue fibre represents a differentiated product derived from the mucinous ground substance. Insofar as is known, elastic fibres have no direct relationship to cells.

COLOUR PLATE

Fig 1

Branch of an essentially normal renal artery of man The internal elastic lamina is 'bathed' in a mucinous matrix (blue), and a similar substance is present between muscle cells of the media Stain colloidal *iron* × 160

Fig 2

Normal aorta of a rhesus monkey The unstained elastic tissue is surrounded by a metachromatic-staining mucinous matrix Smooth muscle cells with bluish cytoplasm are interspersed between the elastic fibres Stain toluidine blue × 700

Fig 3

Coronary artery of man showing mild intimal thickening of a mucinous character (below) A similar mucinous substance is present between muscle cells of the media Bundles of collagen fibres (red) are seen in the adventitia Stain colloidal iron × 160

Fig 4

Abdominal aorta of a pyridoxine-deficient monkey. The intimal plaque is composed dominantly of a mucinous material (blue) There is also an excess of such substance in the media Stain colloidal iron × 15

Fig 5

Abdominal aorta of pyridoxine-deficient monkey The section which is one adjoining that shown in figure 4 has been stained with aldehyde fuchsin which, we believe, reacts with sulphated mucopolysaccharides, staining violet This stain also sharply delineates elastic tissue Stain modified Gomori aldehyde fuchsin × 15

Fig 6

Iliac artery of a pyridoxine-deficient monkey The large intimal plaque exhibits a marked accumulation of blue-staining mucinous material in which there is some cellular proliferation Relatively large pools of mucinous substance are also present i the media Collagen in the adventitia stains red Stain colloidal iron × 80

Fig. 7

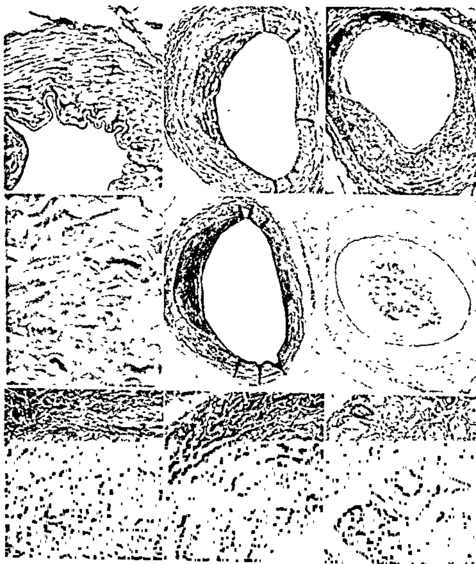
Small artery from a case of diabetes showing swelling of mucinous ground substance and cellular proliferation in the thickened intima. Stain· colloidal iron and Schiff reaction. × 8

Fig. 8

Another small artery from the same source as that shown in figure 7 The sulphated mucopolysaccharide stains violet with this technique. There is also an excess of ground substance in the media The internal elastic layer is clearly shown Stain· modified Gomori aldehyde fuchsin × 80

Fig. 9

A section of human kidney from a case of essential hypertension The high mucinous content of the hyperplastic intimal thickening of the small artery is evident Arterioles are greatly thickened and narrowed Stain colloidal iron × 160



1	4	7
2	5	8
3	6	9

The mucinous substance of the arteries undoubtedly serves as an important element in maintaining the structural and functional integrity of the vessel wall It might be looked upon as a 'mobile cement substance' which binds the fibrillar and cellular components, yet allows them freedom to glide upon one another as the vessel expands and contracts

Structure of Normal Arteries

A brief review of the normal structure of arteries is pertinent to consideration of problems related to the histogenesis of arteriosclerosis or other forms of vascular disease The large arteries such as the aorta and its primary tributaries are of the 'elastic' type In such vessels elastic tissue is present throughout the media. We find that the elastic tissue fibres are bathed in a matrix of ground substance. Smooth muscle cells lie in the spaces between the elastic fibres. These relationships are well shown in the section of monkey aorta stained with toluidine blue (Fig 2) In muscular arteries such as the renal and coronary arteries, a single elastic tissue lamina is seen separating the media and the normally thin intima (Figs 1 and 3). This or other elastic tissue is encased in a 'bath' of mucopolysaccharide The muscle cells of the media also appear to be bound to one another by such material which is sharply stained by the colloidal iron technique (Figs. 1 and 3) A few delicate elastic-like fibres may be seen in the media of such vessels. The adventitia of both the elastic and muscular arteries shows irregularly distributed elastic fibres interspersed between the collagenous bundles of this layer. The basic structure of the muscular arteries appears to be essentially the same irrespective of the size or site of the vessel.

Basic Alterations in Arteriosclerosis

The early structural alterations of a chronic disease such as arteriosclerosis are important in an evaluation of the histogenesis and pathogenesis of the process. It is our belief that alterations in the mucinous ground substance are of fundamental importance in the evolution of arteriosclerosis Observations and illustrations of the process are derived from human arteries as well as from vessels of monkeys subjected to pyridoxine deficiency. The basic alterations appear to be similar

Arteriosclerotic Lesions of Pyridoxine Deficiency in the Rhesus Monkey

These lesions have been described and will be briefly reviewed (Rinehart & Greenberg, 1949, 1951). In monkeys subjected to protracted deficiency of pyridoxine (vitamin B_6) distinctive alterations occur in the



Fig 10 *Fig. 11*

Fig 10.

Segment of abdominal aorta of a pyridoxine-deficient monkey The thickened intima (upper half of section) stains with aldehyde fuchsin, and it would appear that elastic tissue is differentiated in the mucinous plaque The elastic fibres of the media are sharply outlined Stain aldehyde fuchsin × 80

Fig 11

Branch of a renal artery from a pyridoxine-deficient monkey. This section stained only with hematoxylin, was photographed by phase microscopy The mucinous material blends with the more sharply shown elastic fibres of the internal elastic lamina New and imperfectly formed elastic fibres are present in the thickened intima Stain hematoxylin × 600

abdominal aorta and in the iliac and femoral vessels as well as in the smaller muscular arteries of the viscera. The distribution of the lesions is quite similar to that in man. In fact, the character and distribution of vascular lesions in the pyridoxine-deficient monkey is much more analogous to that seen in man than is the experimental cholesterol sclerosis of rabbits

The essential nature of the process is similar in arteries of various sizes. The initial alteration involves a swelling (or increased deposition) of the mucinous 'ground substance'. This is most evident in the intimal zone, but in the more severe lesions there is also a swelling of the ground

substance of the media (Figs 4, 5 and 6). The intima may become considerably thickened with increasing accumulation of mucinous material Concomitantly, there is a proliferation of cells in the intima In such lesions fibrillar material, some with the staining properties of collagen and some of the nature of elastic tissue, is demonstrable in the mucoid plaque (Figs



Fig 12

An iliac artery of a pyridoxine-deficient monkey The thickened intima exhibits marked metachromasia stained with toluidine blue There is also cellular proliferation in the intimal plaque Stain toluidine blue × 80

Fig 13

A section adjacent to that shown in figure 12, stained to demonstrate elastic tissue It is evident that irregular and imperfect elastic tissue has formed in the mucinous matrix of the plaque Stain Weigert-van Gieson × 80

10, 11, 12 and 13). It seems most probable that both the collagenous and elastic tissue fibres are differentiated products derived from the abnormal mucinous ground substance. We have accumulated additional evidence in support of this concept, which is as yet unpublished

Lipid

A detailed study has not been made of lipid in the experimental vascular lesions However, lipid is demonstrable in the more advanced lesions In some instances it appears that lipid may have combined with the mucopolysaccharide In figures 14 and 15 adjacent sections embedded in carbo-

wax (Rinehart & Abul-Haj, 1951 b) have been stained with Sudan IV in one instance and toluidine blue in the other. It appears that the lipid staining reaction with Sudan is dominantly at the sites of concentration of the mucopolysaccharide, which exhibits a deep metachromatic staining

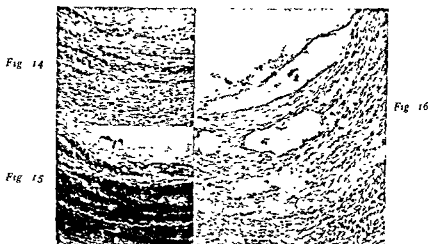


Fig. 14

Segment of an iliac artery of a pyridoxine-deficient monkey The tissue was embedded in carbowax and stained with Sudan IV The dark segments in the upper part of the photograph are stained with Sudan The sites of this staining appear to correspond to the areas of deepest metachromasia shown in figure 15 Stain: Sudan × 80.

Fig 15

A section adjoining that shown above (Fig 14) This section also was embedded in carbowax and was stained with toluidine blue The zones of the intima showing deepest metachromasia appear to correspond with the Sudan staining zones shown above Stain: toluidine blue × 80.

Fig 16

Iliac or femoral artery of pyridoxine-deficient monkey stained with Sudan. There is marked thickening of the intima The dark particles lying in the pools of mucinous material represent lipid stained with Sudan It is probable that some lipid was lost in preparation of the section Stain. hematoxylin and Sudan × 80

reaction This observation suggests that the mucopolysaccharide may have a positive affinity for lipid, as was postulated by Schultz (1922) and by Faber (1949) Accumulation of lipid in the mucinous substance is also shown in figure 16 In other instances, fine lipid droplets may be seen in the cytoplasm of spindle-shaped cells in the deeper layers of a plaque.

Human Arteriosclerosis

Human blood vessels have been studied by similar methods in a considerable series of cases with particular attention being directed to the coronary arteries (Moon & Rinehart, 1952). It is of interest that the



Fig 17 Fig 18

Fig 17

Coronary artery of a female infant of 26 months showing early intimal sclerosis The dark thickened intimal zone stains violet and contains newly formed elastic tissue Fine elastic fibres are also visible in the media Some coarser irregular elastic fibres are seen in the adventitia Stain aldehyde fuchsin × 80

Fig 18

Coronary artery of a man aged 27 stained with aldehyde fuchsin to demonstrate the sulphated mucopolysaccharides and elastic tissue The 'sclerotic' intima about equals the media in thickness The dark-staining mucinous matrix contains newly formed elastic tissue fibres There is an evident excess of mucinous material in the media also Stain aldehyde fuchsin × 80

inception of arteriosclerosis, at least in the coronary arteries, occurs at an early age Mild thickening of the intima is not uncommon in the first decade and shows a progressive increase up to the fourth decade and a somewhat more gradual increase thereafter These changes represent the average However, it is well known that in some individuals sclerotic changes may be advanced to the point of practical occlusion in the third decade, while others may show relatively minor changes in the seventh or eighth decade.

The earlier alterations in human blood vessels appear to be essentially the same as those we have described in the experimental disease of monkeys (Figs 3, 7, 8, 9, 17 and 18)

It is of considerable interest that Virchow (1856) found the earliest changes of arteriosclerosis to occur in the intima and to consist of a 'gelatinous swelling' of this layer. He noted that the accumulated material resembled 'mucus' both chemically and morphologically and observed the presence of delicate elastic fibres in this "structureless, diaphanous, inter-

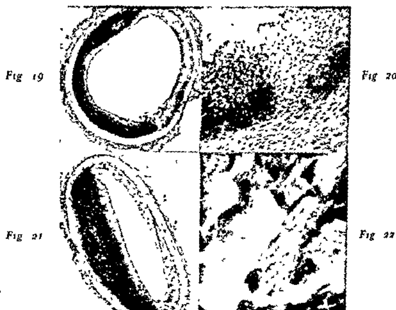


Fig 19
Human coronary artery stained with toluidine blue and Sudan IV. It should be noted that while the intima is almost uniformly thickened, only one segment contains lipid (black in photo) Stain toluidine blue and Sudan × 15

Fig 20
A higher magnification of a segment of the vessel shown in figure 19 The zone to the right shows metachromatic staining with toluidine blue The segment at the left of the same thickness contains lipid Stain toluidine blue and Sudan × 80.

Fig 21
This human coronary artery shows a rather advanced occlusive lesion At the left side of the vessel much lipid has accumulated The lighter-stained portions of the intima exhibit metachromasia with toluidine blue but do not contain lipid Stain: toluidine blue and Sudan × 15

Fig. 22.
Segment of a sclerotic lesion of a human aorta showing undissolved, faintly outlined cholesterol crystals This tissue was embedded in carbowax. Stain: toluidine blue and Sudan × 720.

cellular substance" Concomitant with this alteration, he noted that the connective tissue cells enlarged and multiplied With more refined methods of study we can verify this observation but can say little more. We may speak of deposition or swelling of sulphated mucopolysaccharides in the



Fig 23 Fig 24

Fig 23
Kidney of a pyridoxine-deficient monkey showing hyperplastic thickening and 'elastification' of the intima of a small artery The essential similarity of this lesion to that of the human kidney shown in figure 24 is evident Stain· aldehyde fuchsin × 160

Fig 24
Human kidney In the center is a small sclerotic artery The darkstaining thickened intima 'reacts' with the aldehyde fuchsin stain, and newly formed elastic tissue may be seen At the top of the photograph part of a larger artery is also shown The deeply staining mucinous substance of the thickened intima here is also stained sharply with the aldehyde fuchsin Stain aldehyde fuchsin × 80

intima associated with cellular proliferation, elastification and deposition of collagen. Another alteration seen rather early in the process is focal degeneration of the internal elastic layer at the sites of developing plaques. This is not surprising if one looks upon the elastic tissue as having been derived from and probably maintained by the surrounding ground substance.

With progression of the sclerotic process, increasing amounts of collagenous tissue are found in the plaques, particularly in the deeper layers; the advancing intimal aspect of the plaque is more like the early lesion, presenting the loose mucinous structure rich in mucopolysaccharide. The internal elastic layer shows increasing fragmentation and reduplication

Lipid

In our study of the coronary arteries, no direct or causative relationship of lipid to the early lesions could be shown utilising the usual methods for its demonstration In some instances the intimal layer may show a moderate diffuse thickening, and only one segment may be infiltrated with lipid (Figs 19 and 20) Such a finding strongly suggests that the lipid infiltration is a secondary process.

In the more advanced lesions of coronary arteries lipid is practically always present and is found in the deep layers of the plaque. This lipid may have been imbibed from the blood or may have been derived from simpler substances such as acetate and, being deposited in a site of such low metabolic exchange, it persists, continues to accumulate and acts as an irritant to further augment the sclerotic process (Fig. 21). Frequently cholesterol is demonstrable in such atheromatous masses In sections embedded in carbowax the typical undissolved crystal forms may be seen (Fig 22). Hyaline changes in the partly collagenised ground substance, calcification and increasing accumulation of lipid characterise the more advanced stages of the process.

While the coronary arteries appear to be particularly vulnerable, essentially analogous changes occur in other muscular arteries of similar size.

Small Arteries

It is of some interest to consider the lesions seen in smaller muscular arteries While an extensive study has not been made of such vessels, it seems evident that the pathologic process is similar to that which occurs in the larger vessels, and that here again the experimental lesions are quite like those occurring in man (Figs. 7, 8, 9, 23 and 24) The fundamental lesions involve an increased accumulation or swelling of ground substance, particularly in the intima Accompanying this alteration there is cellular proliferation and deposition of collagen and elastic tissue fibres. Lipid accumulation is less marked The reaction in the small vessels is likely to be diffuse rather than in plaques characteristically seen in larger vessels

Discussion

Limitations of space do not allow full review of the literature pertaining to the connective tissue ground substance of blood vessels. The

early observation of Virchow has been noted Bjorling (1911) drew attention to the 'mucoid connective tissue' of blood vessels and noted its increase in arteriosclerosis Schultz (1922) further directed attention to alterations in the mucinous ground substance of arteries in the development of arteriosclerosis He noted the intimate relationship of the mucinous ground substance to elastic tissue and held the view that elastic tissue was probably derived from the ground substance. Our own observations would support this concept Schultz also drew attention to the affinity of such substances for lipids and calcium, calling attention to the similar property of cartilage.

The derivation of ground substances has not been clarified Asboe-Hansen (1950) noted the close association of hyaluronic acid and mast cells in dermal connective tissues and postulated that the mast cells released this substance He noted, however, that mast cells are not found in relationship to mucinous ground substances containing chondroitin sulphuric acid such as cartilage. The ground substance of arteries is also a sulphated mucopolysaccharide (Meyer & Rapport, 1950), and mast cells are not found in relationship to it. To the writer it seems highly probable that the ground substance of blood vessels in an 'extracellular' secretory product of the component cells of the blood vessels, namely fibroblasts and smooth muscle cells Further, it would appear that elastic tissue as well as collagen fibres are formed in and are differentiated products of the ground substance The manifest increase in the volume of mucinous material in arteriosclerotic blood vessels may be due to an imbibition of fluid by an imperfect, possibly depolymerised ground substance

If, as it appears, the basic lesion of arteriosclerosis involves the ground substance of arteries, it is naturally pertinent to direct attention to factors which are concerned in its formation and metabolism. In view of the experimental evidence presented, there can be little doubt that pyridoxine is so concerned. It is not known whether deficiency of this factor contributes to the pathogenesis of arteriosclerosis in man. While manifest pyridoxine deficiency rarely, if ever, is seen in man, we do not know whether or not mild deficiency states operating over a period of years would exert a deleterious influence Likewise, it is possible that other nutritional or metabolic faults might also impair the formation and maintenance of the vascular ground substance. It should be recalled that arteriosclerosis is a chronic disease with its beginnings demonstrable in childhood and, in the adult, some degree of arteriosclerosis is practically universal

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Summary

The fine structure of normal arteries is briefly reviewed. The alterations seen in human arteriosclerosis and the similar changes occurring in monkeys subjected to protracted pyridoxine deficiency are described and illustrated It is pointed out that swelling of the mucinous ground substance is a prominent feature of the experimental disease and also probably constitutes the basic lesion of arteriosclerosis

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GENERAL CONSIDERATIONS ON COLLAGEN DISEASES

PAUL KLEMPERER
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A PERUSAL of the recent medical literature seems to indicate that the term "collagen disease" enjoys considerable popularity One who has been somewhat responsible for the introduction of the name might consider the frequent references as evidence of approbation by the medical profession But his satisfaction fades when he realizes that the term is not used in the sense in which it was originally proposed. He feels responsible for the misapprehension which he regards as an indictment for not having adequately expressed his ideas He welcomes, therefore, the opportunity of clarifying the original purport of the term, and of amplifying its meaning in the light of subsequent experiences For this purpose it seems appropriate to review first the observations and thoughts which have led to the introduction of the term

Schade (1923) in the first decades of the century called attention to the loose connective tissue, specifically to its extracellular components, as a diffusely distributed colloidal system of biologic importance He recognized its paramount role in water metabolism and in the maintenance of ionic equilibrium and referred to it as "connective tissue organ" Klinge (1933) first realized the significance for pathology of structural changes of the intermediate substances of the connective tissue His investigations of the pathologic anatomy of rheumatic fever and rheumatoid arthritis led him to the conclusion that these maladies are characterized morphologically by a primary generalized alteration of the connective tissue, manifested by mucoid swelling and fibrinoid degeneration of the intercellular material Similar changes had previously been reported by Gerlach (1923) in experimental protein hypersensitivity and these observations were confirmed

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by Klinge. By correlating the results of studies of the living and the dead with those of the experimental animal Klinge concluded that hypersensitivity to streptococci was the etiologic factor in rheumatic fever and rheumatoid arthritis This thesis, also arrived at by Swift (1940), led to an ever-increasing number of microbiologic investigations in search for the antigenic factors of streptococci in general and group A hemolytic streptococci in particular, *immunologic* and epidemiologic studies of patients afflicted with these maladies, and experimental attempts to reproduce the pathologic-anatomic changes of the diseases in animals

Klinge had singled out the fibrinoid alteration of the connective tissue as the most significant structural criterion of the hypersensitivity state and, therefore, extended his pathogenetic hypothesis of rheumatism to a series of human diseases in which microscopic investigations had disclosed the existence of such connective tissue damage Hence, periarteritis nodosa, *thrombo-angiitis obliterans, dermatomyositis, endocarditis lenta, malignant* nephrosclerosis, and certain nephritides were designated by him as *diseases with an allergic background*. When Masugi & Ya Shu (1938) discovered fibrinoid changes in generalized scleroderma, this malady also was included.

Microscopic studies of the organs of patients dying of systemic lupus erythematosus revealed the presence of widespread fibrinoid connective tissue changes in a significant frequency. It was tempting to apply the same pathogenetic interpretation and many authors have subsequently done so (Teilum, 1945, Rich, 1947) However, for several reasons Pollack, Baehr, and I (1941, 1942) could not accept this attractive explanation The course of systemic lupus erythematosus does not reveal clinical manifestations pointing to a *state of hypersensitivity in the strict sense of the term* Furthermore, local fibrinoid connective tissue damage is observed in a variety of morbid conditions where the hypothesis of allergy cannot be seriously entertained, such as in the base of peptic ulcers, in the vicinity of pancreatic necrosis, or in acute bacterial infections It is conspicuous in the vasculature of animals made hypertensive by the Goldblatt mechanism or by desoxycorticosterone, *of dogs treated with repeated adrenalin injections*, and can even be produced by simple squeezing of the skin of the rat

It seemed evident that the diversity of situations in which fibrinoid connective tissue damage was observed could be opposed to the validity of Klinge's pathogenetic generalization. Moreover, while we did not deny that fibrinoid *connective tissue changes occur* in hypersensitivity, the correlation was merely empirical and revealed neither the nature of the tissue

alteration nor the mechanism of its production. Hypersensitivity is too complex and obscure a biologic state to serve as a final explanation for an equally ill-defined morphologic phenomenon. However, we fully accepted Klinge's basic premise that the widespread connective tissue alteration observed in so great a variety of human disease was of significance and that its comprehension would advance the understanding of an etiologically obscure area of human pathology In order to come to such comprehension it first seemed necessary to bring the problem into sharp focus Klinge had recognized that the primary connective tissue alteration in rheumatic fever concerned the intercellular components and that the characteristic cellular abnormalities (Aschoff body) were only secondary Similarly, in systemic lupus erythematosus and in generalized scleroderma the changes of the intermediate substances seemed to us far more meaningful than the associated cellular reactions In our microscopic analysis of the organ changes in lupus erythematosus we were more impressed by the fibrinoid changes of the connective tissue than by its less conspicuous swelling which, only subsequently, was recognized to be the result of an increase of metachromatic substance Following the concept of Neumann (1880) the fibrinoid connective tissue damage was interpreted as a degenerative change of the collagen fibers Accordingly, we believed that the basic pathomorphologic feature was an abnormality of the collagen fiber which we assumed to be due to a physicochemical alteration of the organic compound collagen We were cognizant of the existence of an amorphous ground substance as the second component of the extracellular material of the connective tissue and considered its simultaneous implication in the pathologic process, but we selected the fiber alteration as the conspicuous and more significant morphologic feature The obvious fiber augmentation in generalized scleroderma associated with fibrinoid alteration supported this selection. These considerations led to the choice of the term "collagen disease" to collect descriptively into a group such maladies which were characterized morphologically by systemic alterations of the intermediate substances of the connective tissue, conspicuously of the collagen fibers. In discussing the rationale of the term and of the concept we stated that we did not imply to submit a pathogenetic definition, in fact, the idea was proposed mainly in order to call attention to the significance of the connective tissue as the site of morbid changes and to invite investigations of the reasons for its alteration

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progressing, the discovery of the spreading factor by Duran-Reynals (1942), of hyaluronic acid by Meyer (1947), and the electron microscopic studies of Schmitt et al (1942) stimulated interest in basic investigations of the intercellular components of the connective tissue. Identification of hyaluronidase with the active principle of the spreading factor revealed enzyme-substrate relationships and incontrovertibly established the reality of the connective tissue ground substance The study of the biology of the connective tissue became the focal point for workers from diverse fields such as chemistry, physiology, and histology

There is no question that great advances have been made in the chemical identification and enzyme relations of the various mucopolysaccharides which enter into the formation of the ground substance, but the protein moiety is still largely unexplored. The ultrastructure of collagen has been successfully analyzed by X-ray diffraction and electronmicroscopy, but its relationship to the ground substance has not yet been clarified. The diversity of action of different hormones upon the connective tissue has been amply demonstrated, but the actual mechanism of this influence has not yet been fully established This is particularly true for the role of the adrenocortical hormones which are believed to affect, specifically, morbid conditions of the connective tissue (Hench et al, 1949). Do they act directly upon the cellular elements, the ground substance, or upon the fibers, or rather indirectly upon the metabolism of the chemical precursors? Due to the renewed interest in the connective tissue, old histologic and histogenetic problems have been reinvestigated with newly developed techniques. The formation of collagenous fibers by fibroblasts has been demonstrated by tissue culture and electronmicroscopy (Porter, 1952). On the other hand, it has also been shown that collagen fibers can be reconstituted from a fiber-free solution of collagen without cellular interaction (Nageotte, 1931, Highberger, Gross & Schmitt, 1951). These observations are of importance because they indicate that the formation of intercellular components of the connective tissue might not be under exclusive cellular control. This possibility may also apply to the homogeneous ground substance because even most recent investigations have not provided unequivocal proof for the popular belief that it is manufactured by fibroblasts or other cells (mast cells) Observations of pathology, on the other hand, indicate that abnormal intercellular components of the connective tissue (paramyloid) are deposited without interaction of local cells and are apparently directly transferred from the blood plasma It might well be asked whether

a similar mode of origin should not be thought of for normal constituents of the ground substance (Mueller, 1950, Klemperer, 1953-54).

In appraising the present state of our knowledge of the connective tissue, it must be admitted that certain basic questions regarding the biology of the intermediate substances have not yet been answered; in fact, some have not even been seriously asked. Since the present information regarding the normal state of the connective tissue is still superficial, it is obvious that it cannot suffice to throw light upon the factors which are responsible for its morbid alterations Ought we, therefore, postpone attempts at pathogenetic explanations of the "collagen diseases" until the nature and reactivity of the connective tissue has been fully clarified? Is there really, as Mirsky (1952) expresses it, "a danger of introducing such a term as 'collagen disease' when there is so much that is yet to be done in clarifying what we are talking about!" Ought we to stop at the mere descriptive characterization of these obscure maladies and leave to basic science and the experiment the ultimate task of pathogenetic definition? Can we not expect that persistent investigation and reasoned interpretation of the pathologic manifestations characteristic of the individual members of the group will disclose facts which will give us added insight into the biology of the connective tissue in health and disease? Since the concept of "collagen disease" originated in morphological observations, one might justly inquire whether investigations of anatomic pathology during the past twelve years have contributed new observations which have broadened the concept.

It has already been stressed that the idea of collecting a group of diseases under a unifying term was founded upon the recognition that these maladies show generalized alterations of the connective tissue upon microscopic study The most significant phenomenon is "fibrinoid degeneration" of the collagenous tissue. A clear comprehension of this connective tissue change seems to be the indispensable foundation for a rational inquiry into the pathogenesis of the entire group. The concept originated with Neumann (1880) who believed that the appearance of the connective tissue in certain morbid conditions was the result of a transformation of the collagen fibers into fibrin-like material. This original opinion of Neumann was questioned when Klinge maintained that the microscopic alteration was due to a deposition of a fibrin-like substance between and within the collagenous bundles and not to their chemical transformation This interpretation was promoted by the investigations of Altshuler & Angevine (1949) who concluded that the homogeneous ground

not the fiber, was the anatomic site of the connective tissue alteration, and that the fibrinoid deposits were the result of a *precipitation of mucopolysaccharides* by the action of a basic protein Chemical analysis by Bien & Ziff (1951) of the *fibrinoid substance found in subcutaneous nodules in* rheumatoid arthritis has proven that it does not contain proline and *hydroxyproline and is, therefore, not likely to have originated from collagen* which is characterized by a high hydroxyproline content. A definite chemical identification of the fibrinoid material in rheumatoid nodules was, however, not accomplished by these studies. In previous not published work, Feitelberg in our physics laboratory investigated such material, as well as endocardial vegetations of systemic lupus erythematosus, with wide angle X-ray diffraction. He found that in both instances the diffraction pattern was different from that of collagen; although it showed some similarity with that of fibrin, it was only concluded that X-ray diffraction of fibrinoid substances revealed no more than the presence of a mixture of amino acids. It might be added here that electronmicroscopic investigations by Astbury (1950) and Wolpers (1950) did not contribute to the identification of fibrinoid substances, either It seems, however, to be established that the derivation of fibrinoid substance from collagen must be seriously questioned. While the attempt at chemical identification was not abandoned, it became necessary to reconsider the approach

It was again the study of systemic lupus erythematosus which disclosed *microscopic* features which seem to throw light upon the particular nature of fibrinoid connective tissue changes in this malady, and which suggest *some general principles which may guide us in* considerations of the pathogenesis of the entire group of "collagen diseases". These observations took their origin *from our histochemical analysis* of the hematoxylin bodies which showed that they contained depolymerized desoxyribose nucleic acid *derived from nuclear chromatin of mesenchymal* cells. When the significance of the hematoxylin-stained bodies was recognized for the diagnosis of *the disease, the problem arose how to connect* these alterations of cells with those of the intermediate substances In other words, it was questioned whether the fibrinoid connective tissue damage bore any direct relationship to the observed nuclear chromatin changes. Since mesenchymal cells only were affected, one might believe *that the anomaly* of the intermediate substances of the connective tissue was the result of a disturbed function of these cells which have so generally *been regarded* as the manufacturers of these substances On the other hand, the cellular changes and those of

the intermediate substances might be traced to a common remote cause affecting the entire mesenchyme, or they might be entirely unrelated There were hardly any biologic facts known which could help in resolving these speculations Recent histologic and histochemical investigations in our laboratories (Gueft & Laufer, 1954) on lupus material have demonstrated that depolymerization is only the initial phase of a degradation process of nucleoprotein which leads to the formation of hematoxylin bodies In its further progress DNA gradually disappears and the previously hematoxylin-stained bodies become eosinophilic and stain like fibrin On the other hand, the deposits of fibrinoid substance in the vascular wall and other sites show frequently hematoxylin-stained smudges which are Feulgen positive, indicative of the presence of DNA Appraising the results of these recent microscopic investigations in systemic lupus erythematosus, it can be seen that they contribute to a better understanding of the basic connective tissue change, the "fibrinoid" alteration, which is so prominent in this disease. The histochemical analysis indicates that this "fibrinoid" material includes the protein moiety of the degraded nucleoprotein, and this identification ties together the two characteristic features of the morbid histology, hematoxylin-stained bodies and "fibrinoid" alteration of the connective tissue The presence of degraded nucleoprotein within and outside of the vascular lumina strongly suggests that the deposits within the connective tissue are the results of a transfer or seepage of abnormal protein from that circulating within the vessels

In addition to advancing insight into the pathology of a puzzling disease, the observations lead to certain logical conclusions and raise questions which concern the general problem of "collagen diseases" It has been repeatedly stressed that "fibrinoid" alteration is the basic criterion of the systemic connective tissue change which circumscribes the morphologic definition of the group. This narrow descriptive characterization was obviously inadequate as long as the nature of this alteration and the factors responsible for its development remained obscure. One of the objections against the collective term "collagen diseases" was the fact that the morphologic criteria were too general One of the reasons for the ambiguity is the tacit belief that "fibrinoid" alteration of the connective tissue is a well-defined, always identical manifestation of tissue damage (Klemperer, 1953). The fact alone that it occurs in so heterogeneous morbid and experimental situations should force one to think that the similarity of the microscopic appearance might be deceptive and not denote identity. Such

logical conclusions are generally met by the statement (Baehr & Pollack, 1947, Duff, 1952) "that the reaction of the connective tissue has only limited possibilities and that a more or less stereotype response may occur to a variety of noxious stimuli". The identification of the "fibrinoid" substance in systemic lupus erythematosus as degraded nucleoprotein reveals, for the first time, the chemical nature of such a substance. And just this observation demonstrates also that the "fibrinoid" substances in other maladies must be different, because the metabolic disturbance which is responsible for the occurrence of this type of "fibrinoid" substance is unique for systemic lupus erythematosus. The histochemical identification would not have been possible if the "fibrinoid" substance in systemic lupus had not been a labelled protein, and the ultimate chemical identification of the "fibrinoid" in other maladies will meet far greater difficulties. Nevertheless, I believe that this is one of the basic aims of rational research which will help to define the pathogenesis of the individual members of the group of "collagen diseases". It is obvious that both the theories of hypersensitivity, as well as hormonal imbalance (Selye et al, 1949) proposed to be the universal pathogenetic factor in this group, have been challenged by the recent morphologic disclosures.

The other observation which appears to be of general significance beyond the special problem of systemic lupus erythematosus is the circulation of degraded nucleoprotein, an abnormal protein, within the vascular system and its deposition within the connective tissue as a component of intercellular substances The apparent transfer of a plasma partition into the connective tissue is not startling. In systemic distribution a foreign protein is found as component of intercellular material of connective tissue in para-amyloidosis. This paraproteinosis (Apitz, 1950) is always associated with paraproteinemia; in fact, it is maintained (Randerath, 1950) that para-amyloidosis is an anatomical corollary of paraproteinemia Para-amyloidosis is most frequently, though not invariably, associated with plasma cell myeloma and the recent investigations of the blood proteins in this disease show most commonly abnormalities of the electrophoretic and/or of ultracentrifugal molecular pattern. Association of abnormalities of the intermediate substances of the connective tissue with anomalies of the plasma is, therefore, already well known In fact, Ehrich (1952) included para-amyloidosis with the "collagen diseases". This was an interesting and far-reaching conclusion because it illuminated the problem of this group from a new point of view. Systemic alterations of intercellular

connective tissue substances is the fundamental manifestation of the so-called "collagen diseases". Obviously, the widespread deposition of an abnormal protein such as para-amyloid within the connective tissue reflects a profound alteration of the intermediate substance and, therefore, para-amyloidosis belongs to the general family of collagen diseases. However, the fact that here the nature of the connective tissue alteration is clarified, supersedes the necessity of labelling para-amyloidosis with the broad family term. In general, the term "collagen disease" refers only to one descriptive criterion, if the nature of the structural alteration has been clarified, the term becomes too general and consequently the more distinctive name becomes preferable for the disease. This consideration applies also to systemic lupus erythematosus, which can now be defined as a disease characterized anatomically by a systemic implication of the intermediate substances, due to the deposition of degraded nucleoprotein This degradation is the result of a disturbance of DNA metabolism of mesenchymal cells The cause of this disturbance has not yet been established.

The same pathogenetic principle might well be considered for the exploration of the other members of the group Yet in diseases such as rheumatic fever, rheumatoid arthritis, generalized scleroderma, and serum sickness, no attempts have yet been successful at a closer identification of the abnormal intermediate substances of the connective tissue, only the proteinic nature of the "fibrinoid" material having been established. However, there is ample information regarding the constitution of the plasma proteins, especially in rheumatic fever and rheumatoid arthritis This information, derived from serological as well as chemical investigations, leaves no doubt that the plasma of rheumatic fever patients contains a great variety of antibodies to secretory products of group A hemolytic streptococci, obviously foreign proteins, and that in both diseases the plasma protein partition is conspicuously altered Heteroproteinemia, which seems to play so decisive a role in para-amyloidosis and in systemic lupus erythematosus in the provocation of the changes of the intermediate substances, has therefore been also established in rheumatic fever and rheumatoid arthritis Similar information for generalized scleroderma and serum sickness does not yet exist, but the few reports available indicate, at least, the existence of hyperglobulinemia

The microscopic analysis of the arterial changes in systemic lupus erythematosus has also bearing on the problem of necrotizing arteritis The vascular changes characterized by "fibrinoid necrosis" and severe inflam-

matory cell infiltration have mostly been identified with polyarteritis nodosa (Mallory, 1943). The presence of hematoxylin-stained bodies and hematoxylin-smudged fibrinoid material permits of a separation on morphological grounds. Moreover, the recognition stimulated by these investigations, that fibrinoid as it occurs in different diseases must not be accepted as a homogenous substance merely because of similarity in structure and staining reaction, makes one more inquisitive in regard to the nature of necrotizing arteritis in general. This inquiry is germane to the problem of "collagen diseases" because the conspicuous vascular changes so frequent in this group have been singled out, and given rise to the term "diffuse vascular disease" which is often used synonymously. Recent publications (Zeek, 1952) reveal a healthy revolt against pathogenetic identification of the various instances of necrotizing arteritis. The experimental production of such lesions by different mechanisms unequivocally speaks against an indiscriminate identification in human pathology. In fact, the mode of production in the animal and the histologic analysis (Montgomery & Muirhead, 1953) in human disease indicate that the microscopic similarity is only superficial and that the erroneous identification was mainly determined by the implicit acceptance that fibrinoid alteration denoted material and, therefore, pathogenetic identity

The term "collagen disease", as originally proposed, was not designed to be used as a diagnostic symbol which stands for a complex of functional and structural disorders characteristic of morbid entities It merely called attention to the fact that certain diseases with obscure etiology are distinguished by a systemic alteration of the connective tissue, specifically, of its intercellular components. It collected these clinically heterogeneous morbid states into a group because they shared with each other the seat and features of a basic change of morphology. In contrast to preceding theories, the term did not attempt to explain the implication of the connective tissue. However, it reflected a concept which aimed at pathogenetic definition of the individual members of the group Pathologic anatomy must approach this goal by exact determination of the nature of the structural alterations characteristic of the pertinent maladies, medicine by judicious analysis of the clinical manifestations It is obvious that such explorations of the abnormal must rest upon a full comprehension of the normal. However, the present information regarding the biology of the intercellular substances of the connective tissue,—the focal point of the problem of the "collagen diseases"—is still incomplete in spite of the valuable contribution of the

basic sciences within recent years. Many questions, such as the origin of the homogeneous ground substance, the chemical constitution of its protein moiety, its relationship to fiber formation, the mechanism of hormonal and enzymatic control still await clarification The results of such investigations must be integrated with the interpretation of the abnormal states, characteristic of the "collagen diseases". But the rational analysis of structural or functional manifestations of disease will also raise questions and provide significant clues for the comprehension of the nature and role of the intercellular substances of the connective tissue under normal conditions In this way, the concept of "collagen diseases" (Klemperer, 1950) will have served as a stimulus for scientific contemplation of the intercellular substances and the maladies so designated will become a chapter in a conceptual scheme of "intercellular pathology".

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ARTHRITIS

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Introduction

In recent years, emphasis has been placed on the concept that many of the arthritic diseases represent diseases of the connective tissue. This places the nidus of these diseases in the supporting structures of the body apart from the parenchymal tissues When this concept is carefully scrutinized, it becomes obvious that all disease involves the connective tissue and in only rare instances does a process single out tissues of ectodermal or endodermal origin. Despite this criticism, it has seemed wise to foster the idea that the rheumatic diseases are diseases of the connective tissue since the main impact falls on this tissue and, when visceral involvement does occur, the focus of the lesion is in the reticulum between the parenchymal cells. The rheumatic diseases can be divided into those due to degeneration—chiefly the result of wear and tear—and those associated with inflammation

Degenerative Joint Disease

The prototype of the former is known as degenerative joint disease or osteoarthritis. It may be regarded as resulting from use, affects joints which receive the brunt of life or have been weakened by structural defects, trauma, excessive obesity or prior inflammation With age, degenerative changes can be demonstrated in weightbearing joints in many mammalian species as well as in man The morphologic characteristics are always present whereas symptoms are not The reason for the appearance of symptoms in most instances is the development of a small area of inflammation resulting from minor trauma The primary degeneration is in the ground substance of hyaline cartilage which disappears leaving the reticular framework

visible—a process known as fibrillation. The erosion of cartilage extends towards its border and may penetrate to epiphyseal bone. As a reactive phenomenon to this change, there is active growth of primitive cells where cartilage and synovial intima blend in a zone of transitional tissue In this zone, metaplasia to cartilage and bone may occur as well as at the junction of periosteum and fibrous synovial capsule, thus creating the bony spurs or exostoses so characteristic of degenerative joint disease. When hyaline cartilage is completely destroyed, it is replaced by eburnated bone. Granulation tissue is seen infrequently, inflammation is notable by its absence, and there is little change in the synovial lining. The joint fluid is similar to that seen in traumatic arthritis, showing little evidence of inflammation, of depolymerization of the synovial hyaluronate, or of increased protein content (Ropes & Bauer, 1953). The principles underlying the management of this type of arthritis are simple: attempts should be made to decrease the *amount of trauma and, to tide the patient over the period of inflammation* (which is usually short-lived and not self-perpetuating) by the use of mild anti-inflammatory agents.

Inflammation of the Joints

Arthritis may result from a localized or a generalized inflammatory process The causes of inflammation are protean, and an agent causing inflammation elsewhere in the body can and has caused arthritis

Pyogenic Arthritis

The most common pyogenic organisms resulting in joint inflammation are the pneumococci, staphylococci and streptococci, which may be introduced into the joint by direct trauma or metastatically from a blood stream infection Two other organisms are prone to involve joints during a systemic infection—the gonococcus and the meningococcus These all produce in joints reactions similar to those set up in other sites of the body. The host response is characterized by polymorphonuclear invasion and destruction of tissue with abscess formation The joint fluid is increased in amount, the hyaluronate is depolymerized, there is an increased protein content, an increased number of white cells (predominantly polymorphonuclears) and a decreased glucose content. Since the reparative potentialities of cartilage are practically nil, the great danger from these infections is

that of cartilaginous destruction and repair by joint fusion The principles of therapy are simple—adequate treatment with suitable antibiotics before cartilaginous destruction has taken place

Tuberculous Arthritis

The tubercle bacillus can invade joints—as a manifestation of a haematogenous dissemination or as penetration from contiguous tuberculous bone In localities where pasteurization of milk is not widespread, the bovine tubercle bacillus is prone to invade bones and joints but human bacilli have been recovered from tuberculous arthritis. The lesion is indistinguishable from tuberculosis elsewhere with the usual picture of epithelioid cells, caseation and acid-fast bacilli. Cartilage is undermined and may be destroyed. Although tubercle bacilli may be recovered from the aspirated synovial fluid, the diagnostic procedure of choice is a synovial biopsy. The synovial fluid hyaluronate is depolymerized, there is a tendency to an increased white cell count with relatively low polymorphonuclear percentage, an increased protein and a decreased glucose (Ropes & Bauer, 1953) Antibiotic therapy has been quite successful but in many instances is combined with immobilization of the involved joint with the hope of healing by fusion

"Collagen" Diseases

The vast majority of examples of the inflammatory type of arthritis appears in the group in which the inflammation is of unknown aetiology. This includes rheumatoid arthritis, rheumatic fever, disseminated lupus erythematosus (LED) and periarteritis nodosa (PAN).

Aetiology The most attractive aetiologic hypothesis now entertained is one based on hypersensitivity but the offending antigen is poorly if at all defined. In rheumatic fever, it is linked with infections with multiple types of group A haemolytic streptococci (Rantz et al., 1945) Vascular lesions similar to those seen in PAN have been produced in animals with large amounts of foreign protein (Rich, 1946), and similar vessel changes have been seen in man following contact dermatitis (Rytand et al, 1948), and sulfonamide hypersensitivity (Rich, 1946). The fact remains, however, that in an overwhelming majority of patients with any of these three disease syndromes, no offending antigen or immunologic evidence for such an antigen can be detected

Serologic Reactions. The serologic reactions seen in LED—the LE cell phenomenon of Hargraves et al. (1948), and the positive flocculation reactions—cephalin and thymol turbidity—result from abnormal globulins produced in this disease. The LE phenomenon is quite specific but the relationship of this particular protein to the role of hypersensitivity is not clear. A variety of serologic reactions is seen in many patients with rheumatoid arthritis and in some with LED and PAN The agglutination of sensitized sheep cells by a globulin in the serum of patients with rheumatoid arthritis may be an antigen-antibody reaction. It is more likely a nonspecific reaction in which a system which is about to agglutinate is exposed to a factor which will agglutinate many other readily agglutinable particles such as collodion particles or Kaolin (Wallis, 1946 and 1947). The agglutination of group A haemolytic streptococci by the serum of patients with rheumatoid arthritis is a similar phenomenon, requiring two factors (Lamont-Havers, 1954). The first is present in most adult sera and sensitizes the group A streptococci, making them readily agglutinable. The second is similar, perhaps identical to the factor agglutinating sensitized sheep erythrocytes, a substance produced in patients with rheumatoid arthritis which will agglutinate many readily agglutinable systems, in other words, those "ripe" for agglutination Thus, the serologic reactions, which are more or less specific for these diseases, so far have not helped a great deal in our understanding of their pathogenesis. It should be stressed, though, that, in rheumatoid arthritis where most of the phenomena associated with the clinical picture of the disease as well as with its laboratory picture may be reversed by large amounts of adrenal hormones, these serologic reactions tend to persist This would imply that, alone, these protein reactions represent something other than the host response to the disease and may indicate that the disease process itself is implicated in the production of this abnormal protein.

Pathologic Lesions. The primary lesion in LED and in PAN has long been accepted as a vascular one In the past, there have been advocates for a primary vascular lesion in rheumatoid arthritis These theories were not generally accepted until recently when the mounting weight of evidence has convinced many that the primary lesion in the rheumatoid nodule is a vascular one (Sokoloff et al., 1953). The size of the vessels involved in the three disases—rheumatoid arthritis, lupus erythematosus disseminatus and periarteritis nodosa—seems to be different with the smallest involved in LED, the larger arterioles and small arteries in PAN and the smaller arterioles in rheumatoid arthritis. The vascular lesion in rheumatoid arthritis involves, for the

most part, the intima that in PAN the media and that in LED the whole wall of the capillary. The classical fixed tissue type of hypersensitivity (as seen in the tuberculin reaction) probably has a vascular component at its onset When a minor traumatic episode is produced in loose areolar connective tissue, the first visible reaction in the host is a capillary change consisting of exudation of material from endothelial cells followed by margination of leucocytes and ultimately by cell migration to the traumatized area (Zweifach, 1953) Thus, the vascular nature of the primary lesion seen in the above three diseases need not imply that the hypersensitivity is of the circulating antibody type but could as well be of the fixed tissue type in which circulating antibodies may be difficult to determine.

The cellular reaction seen in PAN is the most extensive while the lack of cellular reaction in LED is characteristic. This reaction in PAN consists of polymorphonuclear and eosinophilic leucocytes, while in rheumatoid arthritis, lymphocytes predominate. Fibrinoid necrosis appears in all three syndromes but our understanding of this change is not clear While it has many of the tinctorial characteristics of fibrin, its composition is believed to be by some, altered fibrin, and by others, altered ground substance (Bennett, 1950).

In LED, the lesion may subside, leaving minimal fine scarring as in the "onion skin" lesion of splenic vessels or only a small amount of increased ground substance as in the "wire loop" lesion of the glomeruli. Granulation tissue is notable by its absence, whereas the appearance of haematoxylin bodies—masses of depolymerized desoxyribonucleic acid particularly in lymph nodes—is said to be seen only in this condition. In PAN, scarring appears in the adventitia but the final lesion is a thrombosis of the involved vessel with infarction of the area fed by that vessel. Again, there is no wild overgrowth of granulation tissue. In rheumatoid arthritis, the vascular lesion is evanescent and can be seen only in the early stages. It is rapidly engulfed by a very vascular granulation tissue which seems to grow so rapidly that it undergoes necrosis This necrotic material is surrounded by epithelioid cells in the typical palisading fashion which in turn are encircled by a zone of more adult connective tissue to form the characteristic rheumatoid nodule Several workers have studied the destruction of collagen in the rheumatoid nodule In the fibrinoid from the center of the nodule, Ziff et al (1953) have been unable to detect an increased amount of hydroxyproline, implying that this substance might be present if collagen were broken down Since this is a relatively small molecule, however, it could well hav

diffused from the area In the center of a nodule, intact and broken down, somewhat denatured collagen has been shown to lie side by side as determined by electronmicroscopy (Kellgren et al., 1951). Thus, there is little evidence from these studies to show that collagen is particularly implicated as the offending antigen Collagen in general is a poor antigen and it is only with great difficulty that antigenicity to it may be demonstrated (Watson et al, 1954). Other components of the connective tissues such as hyaluronic acid and several of the chondroitin sulfates, have been tested for antigenicity in animals and in man but none has been demonstrated It should be pointed out that the differences between these materials as they appear in the body and as isolated chemically are great and could well modify their potential antigenicity. The role of the proteins in the mucopolysaccharide protein complexes in these tissues has not been explored and undoubtedly should be.

Rheumatoid nodules characteristically have a predilection for pressure points such as the juxta-articular regions of the olecranon used in raising the body from an armchair and on the occiput in the bedridden patients. Lesions exhibiting a similar histologic structure may also be seen extensively throughout the body including the supporting structures of the viscera. They have also been noted in heart valves which, when the inflammation has subsided, may be scarred and contracted to produce lesions difficult or even impossible to distinguish from the scarred valves of rheumatic fever The nodules of rheumatoid arthritis represent but one phase of this disease The other is the overgrowth of the synovial tissues, presumably beginning with vascular and small nodular lesions These rapidly become obscured by the thickening of the synovial lining folding up in villi and by overgrowth of a moderately adult granulation tissue known as pannus which spreads widely over the joint invading the cartilage, destroying it and penetrating the subchondral plate. The granulations lead to fibrous ankylosis and when enough cartilage is destroyed, bony ankylosis may ensue. The joint fluid in rheumatoid arthritis is frequently increased in amount, the hyaluronate tends to be depolymerized, there is an increased protein content (similar to that seen in infectious arthritis), the leucocyte count is usually elevated with a tendency to an increase in polymorphonuclear leucocytes, and a tendency toward a slightly lowered content of glucose (Ropes and Bauer, 1953). The synovial fluid in LED and PAN has not been studied in the adequate detail required to make statements concerning its composition.

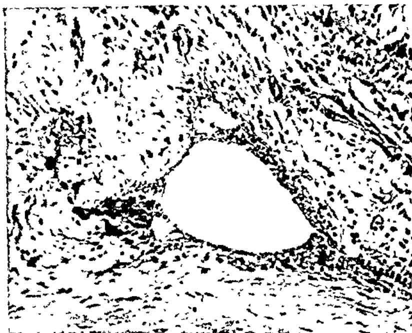


Fig 1.
Fibrinoid necrosis of wall of blood vessel at base of mitral valve in patient with rheumatoid arthritis

Clinical Features A very characteristic feature of these three diseases is that they are self-perpetuating This is difficult to reconcile with an aetiology of hypersensitivity unless one brings in the concept of antibodies to a homologous tissue or tissues as exemplified by progressive chronic nephritis induced by a single injection of an anti-kidney serum This is sheer speculation at the present time but does serve to introduce the concepts of management utilized in these diseases The hypersensitivity, if it exists at all, is of the fixed tissue type and is not influenced by antihistaminics There is no universally accepted form of therapy as with penicillin in gonococcal disease The course of all three of these diseases may be unpredictable In general, it may be said that PAN usually follows a progressive downhill course ending fatally when the blood supply to a vital area is compromised, although long periods of remission may occur. Although LED commonly runs a similar course, spontaneous remissions have been observed which have persisted for months or years

When one considers rheumatoid arthritis, the course is extremely variable This feature of the disease has been recognized only in the past decade and when taken into consideration, the evaluation of therapy becomes difficult. Much treatment proposed in the past must be discarded, as remissions ascribed to a given therapy were probably unrelated to it and represented no more than the natural course of the disease There is considerable evidence, though not accepted by all, that gold in the form of a goldsulfhydryl compound will temporarily modify, to the benefit of the patient, the process of rheumatoid arthritis. The mechanism of this beneficial action, if it exists, is unknown and in no way aids in unraveling the mystery of this disease

Anti-Inflammatory Agents. In all other methods of treatment, reliance is made upon the anti-inflammatory properties of certain drugs and hormones. The salicylates have been used in this group of diseases—notably rheumatoid arthritis—since MacLagen in 1876 pointed out the antirheumatic action of willow bark. Recent work has shown that salicylate has an anti-inflammatory action when used in several experimental models of inflammation, but the pathways through which it works are not understood. It has been suggested that a metabolic product of salicylate—gentisate—may be the active principle. Gentisate under certain conditions will inhibit the action of testicular hyaluronidase but evidence to show that this enzyme is implicated in inflammation, either nonspecific or rheumatic, is nonexistent. With the hormones—cortisone (Kendall's compound E), hydrocortisone (Kendall's compound F), and corticotrophin (ACTH)—the anti-inflammatory action is somewhat better understood although the particular enzyme system blocked is not known. These three hormones have a common result in the intact animal, hydrocortisone working at the tissue level, cortisone probably converted to hydrocortisone in the body and corticotrophin acting through the adrenal to produce a substance similar to, if not hydrocortisone itself. Since the three hormones may be assumed to act through hydrocortisone, this will be used as the example It is thought that it exerts its anti-inflammatory action by blocking the elaboration of a substance necessary for the maintenance of the inflammatory process produced by damaged cells. This substance causes changes in the vessel wall leading to margination and may be chemotactic for the cells required for the completion of inflammation. The processes of repair, of turnover, of maintenance of structure, and of growth are intimately connected with the elaboration of this substance, each being slowed during the administration of this hormone Following with-

drawal of the hormone, the phenomenon of the rebound is often seen, and can be illustrated most graphically with growth curves where, during hormonal administration, the growth rate is slowed and, following withdrawal, accelerated beyond the control figures. The rebound has been recognized

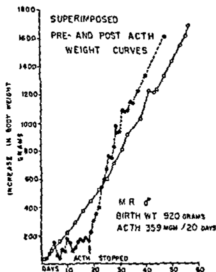


Fig 2
Example of "rebound" weight gain following withdrawal of ACTH
For 12 days following cessation of treatment rate of gain exceeded pretreatment rate. Following this pretreatment rate was resumed. Base-line weight for pre-ACTH curve (open circles) is 1270 gm, that for post-ACTH curve (solid circles) is 2160 gm

clinically in hormonal withdrawal in the rheumatic diseases (Fletcher et al., 1952) although it cannot be as well documented as in growth curves. If the first premise is correct, namely, that a substance is not elaborated by damaged cells during cortisone administration, it might be assumed that precursors to this material may be stored up and accumulate. Following hormonal withdrawal, this accumulated material is suddenly utilized and an acceleration of the inflammatory process—the rebound—takes place. In clinical practice, complete suppression of the inflammatory process is seldom feasible since doses of hormone required to do this would be incompatible with life. The phenomena associated with withdrawal may continually be taking place during the clinical use of these hormones. The inflammatory process in rheumatoid arthritis which is blocked by cortisone

requires a period of time to build up to the level at which clinical symptoms appear Thus, it is possible that the rebound phenomenon may be continually going on at a level insufficient to produce the clinical symptoms used to gauge the amount of hormone administered In this way, one might explain the acceleration of the process at the tissue level coexistent with suppression of clinical symptoms. This type of reasoning has been used to explain the apparent increase in visceral involvement seen in rheumatoid arthritis since the advent of the cortisone era (Robinson et al, 1953).

Summary

In summary, the rheumatic diseases involving tissues of mesenchymal origin may be roughly divided into two groups: those resulting from degeneration and the inflammatory type. The aetiology of the inflammatory *arthritic diseases is understood in some but in the majority is not and must* at the present time be approached through speculation Present hypotheses favor the basis of hypersensitivity of the fixed tissue type bringing in the concept of autoantibodies to explain the self-perpetuating nature of these syndromes. In the therapy of nonmicrobial inflammatory arthritis, resort is made to the antiphlogistic agents such as the salicylates and the 11-oxy-steroids of the adrenal cortex. From indirect evidence, some concept of the mode of action of these hormones in inflammation may be surmised. Our knowledge of the structure of the tissues involved, the reaction of these tissues to noxious stimuli and the particular noxious stimuli involved in the rheumatic diseases affecting these tissues is limited. Until more knowledge is gained in these areas, we will continue to grope our way in the management of these human diseases on an empirical basis.

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SOME SYSTEMIC CONNECTIVE TISSUE DISORDERS PERTAINING TO DERMATOLOGY

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Introduction

Recent connective-tissue research has introduced a number of new aspects into dermatology. It is now realized that the connective tissue of the skin is part of a system possessing important functions

Until a few years ago, dermatology was external medicine. To-day at least a group of dermatological diseases are considered external only from an internal point of view. Now as previously, the aim is to treat a vital organ which besides other important functions serves as man's front towards environment. But knowledge has been extended, the means have changed, and the therapeutic points of attack have become more numerous.

The skin is a mesenchymal organ with a cover of ectodermal cells with special properties and functions. The corium—or dermis—is made up of fibrillar connective tissue. *Cells* and *fibrils* are embedded in a mucinous matrix or ground substance.

Collagen fibrils, arranged in bundles, constitute the predominant element of the dermal connective tissue. Reticulin fibres, related to the collagen fibres, are particularly ample in the dermo-epidermic junction and in the perivascular areas The elastic fibres, consisting of thin fibrils and threads, form a network, anastomosing with one another.

The fibroblasts predominate numerically among the cells of the dermal connective tissue in an adult body; they are believed to play a part in fibrillogenesis The mast cells are present in large numbers, especially in young individuals i e. in growing connective tissue Apparently, they secrete im-

portant components of the mucinous ground substance, primarily the viscous mucopolysaccharide hyaluronic acid.

In addition to these cells, which appear to be concerned with the new-formation and integrity of a normal connective tissue, the dermis may contain histiocytes or macrophages, plasma cells, and lymphocytes acting in local and systemic defence

In the subcutaneous tissue the predominant cellular element is the fat cell—a mesenchymal cell holding droplets of fat

When the dermal connective tissue is diseased, we are dealing with a skin disease. On the other hand, external factors may primarily attack the epidermis This is true in contact eczemas, but there is probably no dermatological condition that does not—primarily or secondarily—involve the connective tissue.

It seems beyond doubt that the epidermis may change in response to various morbid changes of the underlying connective tissue. For instance, thickening of the epithelial layer and keratosis is a rather constant response to an accumulation of mucin in the dermis, e.g in myxoedema. Hair growth appears to be influenced by changes of the connective tissue, and the same probably applies to the growth of the nails Diseases of the mesenchymal structures are invariably associated with characteristic changes of the surface

The diseases to be mentioned below are indubitably of widely different ætiology and pathogenesis, but all of them have the common feature of affecting the connective-tissue system and of giving rise to morbid changes in the ground substance, fibrils and cells.

This is not the place to deal with all dermatological mesenchymoses In order to draw attention to the significance of the mesenchyme as the soil of disease, I have chosen some systemic connective-tissue diseases whose names are coined by dermatologists, because the cutaneous symptoms predominate the syndrome or are at least signs of essential diagnostic significance

As far as possible, the classification will be based on primary morbid changes in the various components of the connective tissue, but owing to the close functional relationship of the cells and the intercellular substance, such a classification cannot be absolute

Disorders Affecting All Mesenchymal Elements. "Mesenchymal Diseases"

The ætiology of one important group of dermatological connective-tissue disorders to be dealt with below is entirely unknown, and little is known about their pathogenesis. All these conditions affect the mesenchymal system in the extended sense of the term, the reticulo-endothelial system being—at least at some stage of their course—actively involved in the processes. At an early stage they seem to affect primarily the mucinous system (the ground substance and the mast cells). In more prolonged cases the collagen system (precollagen, collagen fibrils, fibroblasts) or the elastic tissue (the elastic fibres) will be involved as well. Certain chemical changes in the intercellular substance, induced by local or systemic actions, such as fibrinoid, hyaline, amyloid, or paramyloid changes, have been demonstrated in certain connective tissue diseases. They have to a marked extent formed the basis of pathogenetic classifications and considerations (pp 178, 196, 251)

Disseminated Lupus Erythematosus

This diagnosis is probably in most cases made in a dermatological department, because the cutaneous signs are practically always present. But the disease affects the connective-tissue system as a whole In the predominant majority of cases, it affects women of the age of sexual maturity (20–45 years of age), and this has drawn attention to the endocrine apparatus. The question of a possible allergic pathogenesis is still of topical interest (Klemperer, p. 252, Teilum, 1948, and others).

The cutaneous signs are of essential diagnostic importance. The affection may bear a certain resemblance to chronic discoid lupus erythematosus. Nevertheless the two diseases may be essentially different, although transitions from one into the other have been reported

The skin lesions may manifest themselves as erythematous, urticarial, bullous, erythema multiforme-like or erysipelatous elements, often associated with dermal hæmorrhages. Sometimes, the eruption starts in the face, in which case it is characterized by a butterfly distribution, covering the nose and cheeks There may be elements with follicular keratosis and atrophy as in the chronic discoid l e Gradually, the eruption spreads to the trunk and extremities (Figs 1, 2) It is often—at least at the outset—localized to

areas exposed to light, but mechanical traumas also appear to play a rôle, the skin of e.g. the heels, elbows and knees being affected with particular frequency. The fingers often exhibit characteristic elements, the dorsal aspects of the phalanges and the nail folds may show vivid to bluish red,



Fig. 1
Systemic lupus erythematosus

oedematous, infiltrated, chilblain-like elements with or without purpura and occasionally with vesicles and bullae.

As far as the skin symptoms are concerned there may be completely symptom free intervals, but such remissions may be accompanied by exacerbations in other organs.

The oral mucosa may exhibit aphthae, mucosal bleedings in the form of petechiae and ecchymoses, or purulent gingivitis. Pluriorificial ulcerative mucosal lesions have been observed.

The disease is accompanied by periods of fever, fluctuating according to the general condition.

Microscopic examination of skin lesions reveals accumulations of a metachromatic, hyaluronidase-sensitive ground substance and mast cells in

the connective tissue of fresh lesions (Altshuler & Angevine, 1949, Asboe-Hansen, 1950 c). In cases of longer standing, metachromasia is less constant and often insensitive to the action of hyaluronidase; and fibrinoid changes are frequently observed. There will be accumulations of fibroblasts

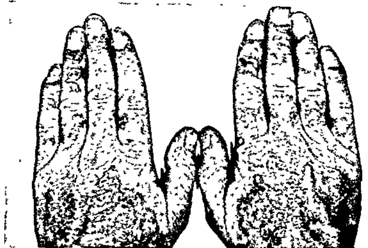


Fig 2
Systemic lupus erythematosus

and increased formation of collagen fibrils, resulting in regular fibrosis In all advanced cases there are signs of degeneration and fragmentation of the collagen fibrils There are infiltrations with lymphocytes, mast cells, and plasma cells, a certain number of macrophages is constantly present. Usually, there is vascular proliferation and intramural oedema In rare cases the vascular changes acquire the character of periarteritis nodosa, showing fibrinoid necrosis as well as inflammatory cellular infiltration.

In all mesenchymal tissues "*hæmatoxylin stained bodies*" have been found, and *fibrinoid degeneration* is a characteristic connective tissue change in all parts of the body

In the blood and bone marrow characteristic "*l e. cells*" (Hargraves et al, 1948) have been observed, a phenomenon that is due to an abnormal protein within the globulin fraction of the blood and certain changes in the nuclei of the white blood cells. Regarding the relationship of the last mentioned three phenomena, the reader is referred to Klemperer, p 256, and Klemperer et al., 1950.

Hyperglobulinæmia is a fairly constant finding, and so is, correspond-

ingly, an accumulation of protein producing pyroninophil mesenchymal cells, including plasma cells in the spleen and other organs.

Anæmia and leukopenia are always present; the latter is of diagnostic value. Articular involvement is among the most constant symptoms of this disease. It may range from arthralgia without objective signs, to large swellings and effusions in the joint cavities. Destruction of cartilage or bone does not occur. The skeletal and cardiac muscles, on the other hand, are often involved, this entails pain and physical fatigue. The serous membranes, the pleura, pericardium, and peritoneum, may be involved. Atypical, verrucous endocarditis, described in 1924 by Libman & Sacks, is an important and fairly constant sign (Mortensen & Gormsen, 1952). Renal symptoms are rather constant, and may be severe. Hæmaturia (microscopic) and albuminuria belong to the ordinary objective signs. The condition may resemble nephrosis and entail oedemas and hypoproteinæmia. Uræmia is a fairly common terminal sign. Hyaline changes are observed in the glomeruli. The spleen is enlarged and periarterial fibrosis is a common lesion. Lymphadenitis with necrotic foci is often present; the nodes are indolent and freely movable.

The prognosis of systemic l.e. is grave; it invariably leads to death.

Treatment: Intensive ACTH or cortisone therapy often gives dramatic results. The cutaneous signs as well as the symptoms from the mucous membranes, joints, serous membranes, and heart may subside in a few days. Also the anæmia, albuminuria, and hæmaturia may subside. The γ-globulin in the serum becomes normalized. Other globulin fractions as well as the l.e. cell phenomenon may remain unchanged. The treatment may be a life-saving measure, it is not, however, devoid of risk. Death from heart failure may occur. Recurrence invariably follows withdrawal of the hormone.

Scleroderma

Scleroderma is a connective-tissue disease that affects the dermal connective tissue and very often the connective tissue in other locations too. As in disseminated lupus erythematosus, the skin need not be the primary site of the disease, but in the great majority of cases it will sooner or later become involved.

Scleroderma may manifest itself as a diffuse, progressive variety or as circumscribed scleroderma: morphea or scleroderma guttata (white-spot disease).

The former is a chronic, progressive systemic disease. In the initial stages, the skin is erythematous, vivid red, or purple, oedematous or infiltrated. Gradually, however, the epidermis grows atrophic and the corium becomes thickened, sclerotic, and firmly adherent to the underlying tissues (fascia etc.). The surface becomes smooth and glistening, whitish-yellow



Fig 3
Scleroderma

in colour The skin loses its normal suppleness, elasticity, and mobility in relation to the underlying structures. The face is expressionless and stiff (Fig. 3). The lips become thin, and radial lines form around the mouth On the nose and ears, the skin is thin and brittle. The skin cannot be folded as normally and on the extensor surfaces of the joints it is tight and thin, often showing trophic ulcerations. The fingers gradually assume a semiflexed position, the pulps being tapered. The skin of the pulps is also easily vulnerable owing to the diminished elasticity; in many cases it is hyperæsthetic, but often almost indolent This condition is termed *sclerodactylia* (Fig 4). Sclerodermic patients have a feeling of being enclosed in a stiff armour, and this feeling has so much to do with reality, that respiration may be impaired by the indurated skin and subcutaneous tissue of the

trunk Calcification of the connective tissue in all locations is not uncommon Sclerodermic skin often exhibits hyperpigmentation as well as depigmentation. In atrophic areas, hair growth ceases.

The condition called *acrosclerosis* is a more localized variety of diffuse scleroderma The face, neck, hands and feet are involved, and the cutaneous changes are often combined with vascular crises resembling Raynaud's disease.

In the early stages, microscopic examination of histologic sections shows an increase in the mucopolysaccharide-containing ground substance, a mucinous oedema Accumulations of mast cells are seen free in the connective tissue and, together with some lymphocytes, in a perivascular situation A good deal of fibroblasts are seen all over the dermis The collagen bundles are swollen. Gradually as atrophy sets in, the cells decrease in number, the ground substance becomes sparse, showing no or faint metachromasia The collagen bundles exhibit sclerosis, becoming homogeneous and densely packed The elastic fibres are atrophic, split up in bits, arranged parallel with the surface in the most superficial layers of the corium Fibrinoid degeneration of the dermal tissue is seldom outstanding, though often present Amyloid depositions occur. The first change in the vascular walls is mucinous oedema involving the intima as well as the media, then fibrinoid changes occur. All layers of the vascular walls grow thickened and fibrosed and their lumina diminished or obliterated. The sweat glands may be immediately surrounded by the fibrous, sclerotic connective tissue. Sebaceous glands and hairs are often absent.

The disease may involve the connective tissue all over the body Skeletal as well as cardiac muscles may exhibit pronounced fibrosis giving rise to myalgia and cardiac failure Changes in the capsular connective tissue entail pain and swelling of the joints. The vascular changes also involve the coronary vessels. There will be more or less marked fibrosis of the liver, pancreas, spleen and lungs Bronchopneumonia is a common complication The kidneys may be fibrotic and show hyaline degenerations of the glomeruli, giving rise to renal symptoms resembling those of disseminated lupus erythematosus. The gastro-intestinal tract is often affected and the patients may suffer from dysphagia and oesophagitis, oesophageal stricture and diverticula as well as hiatal hernia Pericarditis and pleurisy may give serious symptoms too The central nervous system may exhibit degenerations giving rise to neurological symptoms In the bone marrow, there is an increased formation of connective tissue with a decreased erythro-

poiesis and consequent anæmia. Cataract is one of the rare ocular symptoms; episcleritis is not uncommon.

Hyperglobulinæmia may occur, but is seldom an outstanding feature. The reticuloendothelial system is more or less involved in the pathologic reactions.



Fig 4
Sclerodactylia

Scleroderma bears a certain relation to the endocrine apparatus. It attacks the endocrine glands, so hormonal insufficiency may be a secondary phenomenon The disease is definitely more common in women than in men.

Death occurs from intercurrent infections, usually bronchopneumonia.

Treatment with ACTH and cortisone is beneficial in fresh cases and may arrest a progression while the changes of the ground substance are prevailing Regression of the sclerosis and connective-tissue indurations rarely occurs and at any rate only after extremely prolonged treatment The fibrinoid necrosis allegedly remains unchanged

Dermatomyositis

This is a rather uncommon mesenchymal disease that may occur at all ages and in both sexes Frequently, it starts as a rather sharply defined skin erythema, growing oedematous and later on more infiltrated. The face, particularly the eyelids, are often first affected, but gradually the disease

spreads distalwards and the entire skin may become involved. These lesions may subside, but often they persist in the same locations. In advanced stages, the syndrome reminds of diffuse scleroderma. There may also be atrophy of the skin with pigmentations of a reticular pattern and telangiectasis as in poikiloderma vasculare atrophicans.

The microscopic appearances in fresh cases resemble lupus erythematosus, while cases of long standing are microscopically indistinguishable from scleroderma. The inflammatory changes are relatively outstanding.

In the subcutaneous adipose tissue and in the muscles there may be depositions of calcium.

The skeletal muscles are involved, and there is a more or less marked muscular weakness. The muscles are tender, swollen or indolent and puffy, becoming gradually atrophic. *In the skeletal muscles there is oedema, degeneration of the fibres, and cellular infiltration progressing until an atrophic and fibrotic appearance.* Rarely, the cardiac muscle is involved. The gastrointestinal tract, especially the oesophagus, may be involved as in diffuse scleroderma, and intestinal ulcerations have been observed. The serous membranes and the synovial connective tissue may be affected.

An increase in the α- and γ-globulin fractions of the blood serum may be more or less marked. Paramyloidosis and plasma cell accumulation in the bone marrow have been reported (Jorgensen, 1944).

The disease progresses by attacks, accompanied by fever, and leads to death.

Treatment with ACTH and cortisone has shown favourable effects in acute cases, causing regression of the symptoms. Withdrawal is followed by recurrence. Some cases do not respond favourably to hormone therapy.

POIKILODERMATOMYOSITIS

Poikiloderma vasculare atrophicans may occur by itself without other complications, or it may be combined with muscular disorder. It has no site of predilection. *In the early stages it is represented by itching papules of varying size, oedematous infiltrations with a tendency to confluence.* These lesions are often symmetrical. Later they undergo atrophy of the connective tissue followed by telangiectasis and occasionally small cutaneous hæmorrhages. The thin, atrophic areas often show brownish pigmentation of a net-like pattern (Fig. 5). *It is dry without any sebaceous secretion and with negligible scaling.*

The more advanced cases may be complicated by myositis, characterized by weakening and tenderness of the skeletal muscles and a course resembling that of dermatomyositis. The lymph nodes and the spleen may be enlarged.



Fig 5
Poikiloderma vasculare atrophicans

Some cases develop into mycosis fungoides or reticulosarcoma, and death may occur from leukæmia.

The microscopic appearances are dominated by the changes of the connective tissue. The corium appears as a quite thin layer unlike scleroderma It is densely infiltrated with mast cells, lymphocytes, fibroblasts, and histiocytes. In the active stages there is an ample content of mucopolysaccharides in the ground substance The dermal vessels are dilated The collagen bundles are oedematous, degenerated and sclerotic and there may be pronounced degeneration or complete disappearance of the elastic tissue. Hair follicles and sebaceous glands are reduced in number or absent.

The accompanying myositis is characterized by cellular infiltrations, oedema, degeneration, and necrosis of the muscle fibres.

Decalcification of bones may occur.

Treatment with ACTH and cortisone has proved effective in some cases

Serum Sickness

This allergic reaction to foreign protein develops 6–12 days after an injection of serum The initial and most constant sign is represented by skin manifestations. erythema, urticaria or Quincke's oedema, occasionally also small cutaneous hæmorrhages due to increased capillary fragility More rarely, the erythema is morbilliform or scarlatinoid, nearly always itching Frequently, the eruption appears first at the site of the serum injection In rare cases, particularly in relapses, the eruption suggests erythema multiforme. Conjunctivitis is often present. Albuminuria is not uncommon Enlargement of the lymph nodes is a fairly constant sign, the spleen may be enlarged, and lastly articular symptoms are common—in particular following injection of serum in large quantities The joints become hot, swollen, reddened, sometimes showing effusion in the joint cavity All joints may be involved, large as well as small, often including the mandibular joint Severe cases are accompanied by fever Occasionally, there are neurological symptoms, such as headache, optic neuritis, or peripheral neuritis Gastro-intestinal symptoms such as pain, nausea, and diarrhoea may also occur Periarteritis nodosa has been reported to develop in the course of serum sickness (Rich, 1942).

Serum sickness is one of the diseases that are accompanied by fibrinoid degeneration and necrosis of the mesenchymal tissues The allergic genesis is more evident than in any other condition

The prognosis is favourable.

Only symptomatic treatment is needed ACTH and cortisone are effective

Periarteritis Nodosa

This disease is generally interpreted as an allergic manifestation, and typical cases have been reported following injection of foreign protein, ACTH, sulphonamides or other agents. The deep cutaneous and the subcutaneous vessels (arterioles) may be involved as well as those of the gastrointestinal tract, the heart, kidneys, and lungs. The cutaneous symptoms differ widely from case to case and also within the individual case. Subcutaneous nodules are occasionally palpable; cutaneous hæmorrhages

and necroses are less common. On the other hand, efflorescences, such as erythema, papules, and urticaria are relatively frequent.

The microscopic picture is characterized by an increased production of a highly metachromatic ground substance in the subintimal layer, in the media as well as in the adventitia. Fibrinoid changes are often observed An accumulation of mast cells and fibroblasts is an outstanding feature and, moreover, all layers contain polymorphonuclear leukocytes most of which are eosinophil, and a varying number of lymphocytes. The endothelium may be involved, giving rise to thrombosis and formation of granulation tissue resulting in occlusion of the lumen. Multiple aneurysms are formed. The connective-tissue newformation progresses, showing scarring and fibrosis.

Other clinical signs vary according to the extent. The disease may be accompanied by ocular (cf p. 302) and articular symptoms (cf. p 266), abdominal pain, nephritic signs, enlargement of the lymph nodes, and cardiac symptoms if the myocardium is involved. Frequently, there is rather marked eosinophilia in the blood, and in some cases the serum globulin values are enhanced.

The prognosis is bad Most cases lead to death.

Treatment with ACTH and cortisone is effective, and prolonged remissions have been observed after intensive treatment In cases of widespread vascular damage the treatment entails danger of vascular obliteration and infarction.

Disorders Characterized by Primary Changes in the Ground Substance

Of all the constituents of the connective tissue the mucinous system is the most lively and quickest participant in physiological variations. It varies in amount, water content, and chemistry. The fibrils may alter too, but their turnover is very slow. The ground substance, and consequently the connective tissue as a whole, is governed by hormonal factors. Presumably, the hormones act primarily on the cells engaged in forming the intercellular substance *viz* the mast cells and the fibroblasts.

The endocrine apparatus exerts a regulating influence on connective tissue throughout the body. Therefore, hyper- or hypofunction of the endocrine glands will manifest itself in the skin as well as in any connective tissue

Hypothyroidism

In this condition, the skin is dry, keratotic, thickened, and cool It feels elastic, but does not retain impressions of a finger It is pale and yellowish The non-pitting oedema is usually most marked on the eyelids, cheeks, and hands. Hairing is scanty, both on the scalp, in the region of the eyebrows, in the axillae, and in the pubic region Hairs and nails show signs of dystrophy

Microscopic examination has revealed severe changes in the intercellular substance In the amorphous ground substance, the content of the muco-polysaccharides hyaluronic acid and chondroitin sulphuric acid is greatly increased The number of mast cells—nearly always highly granular—is increased free in the tissue as well as in the perivascular areas In extreme cases the collagen bundles and the elastic fibres are burst apart, the collagen fibrils may be swollen, degenerated, and basophil Advanced cases show oedema There is also a loss of subcutanous fat

In most cases any associated articular symptoms—called myxoedematous arthritis—are rather mild, but they may give rise to joint swelling and limitation of movement There is mucinous oedema of the synovial connective tissue as well as the fibrous capsule. The hyaluronic acid content of the synovial fluid is increased (Ropes et al, 1947) As a rule, there are only faint signs of inflammation, and the articular cartilage is never involved

Early arteriosclerosis may be associated with myxoedema in which condition lipæmia and connective tissue changes in the vessel walls are constant signs.

A number of other symptoms changes of the cardiac muscle, hoarseness, etc may be attributed in large measure to the connective-tissue lesions

Treatment Thyroid hormone

Circumscribed Myxoedema

The changes—usually of a pretibial situation—occur in thyrotoxic patients with recurrence following operation for thyrotoxic goitre. The skin and the subcutaneous tissue feel cushiony, soft elastic, in advanced cases firm, hard, surrounding the lower part of the legs in the form of a cuirass-like cuff The surface may be smooth, but it may also show papular, button-shaped excrescences (Fig 6) The skin is cool, but often sweating.

The microscopic appearances remind of myxoedema, but in this con-

dition more advanced and even monstrous degrees are encountered (Watson & Pearce, 1949, Asboe-Hansen, 1950 a). The papillary layer is not involved, and this is a characteristic feature which distinguishes this condition from hypothyroid myxoedema.



Fig 6
Circumscribed myxoedema

The disease is believed to be due to a preponderance of the pituitary thyrotrophic hormone. It often co-exists with progressive, usually malignant, exophthalmos that is interpreted essentially as a consequence of retrobulbar accumulation of a water-binding connective tissue holding large amounts of hyaluronate (cf. p. 143). The pronounced muscular weakness and atrophy encountered in pituitary-thyroid disorders are explicable by intrasarcolemmic accumulations of a substance containing mucopolysaccharides (Asboe-Hansen et al, 1952, cf. p. 144).

The condition shows a certain tendency to spontaneous regression.

It has been treated with varying success with X-radiation of the pituitary gland, with thyroxin, oestrogenic hormone, and cortisone. Treat-

ment with hyaluronidase (Grais, 1949, Asboe-Hansen, 1950 a) is only of academic interest, because although the hyaluronic acid is broken down and subsides, *it rapidly accumulates anew.*

Acromegaly

Acromegaly represents a hyperfunction of the adenohypophysis leading to excess production of growth hormone and thyrotrophic hormone The changes affect primarily the ground substance, but fibrosis is pronounced The skin is coarse, thickened, and wrinkled. All mesenchymal tissues grow. The dermal connective tissue is ample, firm, and fibrous

It is characteristic that fibromas are present in the skin, the hair is coarse, and the epidermis keratotic. The patients are apt to form keloids following injury and also without external traumatization The nails are thick, flattened, and grooved.

Microscopic examination shows signs of lively newformation of connective tissue. *The ground substance is ample showing intense metachromasia*, and there are numerous mast cells and fibroblasts. The bundles of collagen fibrils are thick and coarse The papillae corii are large and broad

The condition is accompanied by arterial enlargement due to connective-tissue proliferation in the walls Muscles are weakened and atrophic and mucin accumulates within the sarcolemma (p. 145) Bones and cartilage are thickened, but longitudinal growth is not increased If hyperpituitarism occurs before the ossification of the epiphyseal zones it may lead to gigantism.

Cushing's Disease

This condition of hypercorticoidism is caused by basophil adenoma of *the hypophysis or by adrenal cortical hyperplasia leading to an increased* production of glucocorticoids The increased cortical activity entails numerous, characteristic changes of the skin The patients are fat, the obesity being localized particularly to the face, neck, and trunk They exhibit hypertrichosis, abnormalities of pigmentation, and acne Constant and characteristic are the purple or whitish striae atrophicae on the abdomen, hips, and shoulders The elasticity and tensile strength of the skin are considerably decreased Dilatation of minor blood vessels, hæmorrhages,

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It has been treated with varying success with X-radiation of the pituitary gland, with thyroxin, oestrogenic hormone, and cortisone. Treat-

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Acromegaly

Acromegaly represents a hyperfunction of the adenohypophysis leading to excess production of growth hormone and thyrotrophic hormone. The changes affect primarily the ground substance, but fibrosis is pronounced The skin is coarse, thickened, and wrinkled All mesenchymal tissues grow The dermal connective tissue is ample, firm, and fibrous.

It is characteristic that fibromas are present in the skin, the hair is coarse, and the epidermis keratotic. The patients are apt to form keloids following injury and also without external traumatization The nails are thick, flattened, and grooved.

Microscopic examination shows signs of lively newformation of connective tissue The ground substance is ample showing intense metachromasia, and there are numerous mast cells and fibroblasts. The bundles of collagen fibrils are thick and coarse The papillae corii are large and broad

The condition is accompanied by arterial enlargement due to connective-tissue proliferation in the walls Muscles are weakened and atrophic and mucin accumulates within the sarcolemma (p 145). Bones and cartilage are thickened, but longitudinal growth is not increased If hyperpituitarism occurs before the ossification of the epiphyseal zones it may lead to gigantism

Cushing's Disease

This condition of hypercorticoidism is caused by basophil adenoma of the hypophysis or by adrenal cortical hyperplasia leading to an increased production of glucocorticoids The increased cortical activity entails numerous, characteristic changes of the skin The patients are fat, the obesity being localized particularly to the face, neck, and trunk They exhibit hypertrichosis, abnormalities of pigmentation, and acne Constant and characteristic are the purple or whitish striae atrophicae on the abdomen, hips, and shoulders The elasticity and tensile strength of the skin are considerably decreased. Dilatation of minor blood vessels, hæmorrhages,

dition more advanced and even monstrous degrees are encountered (Watson & Pearce, 1949, Asboe-Hansen, 1950 a). The papillary layer is not involved, and this is a characteristic feature which distinguishes this condition from hypothyroid myxoedema



Fig 6.
Circumscribed myxoedema

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Disorders Characterized by Primary Changes in the Elastic Tissue

Pseudoxanthoma Elasticum

This is a rare disease affecting to a major or minor extent the elastic tissue of ordinary fibrillar connective tissue, of the elastica of the vessels, and of Bruch's elastic membrane in the retina. The skin is affected mainly on the lateral aspects of the neck, in the flexures of the elbows and knees, and in the axillae, but the lesion may also involve the face In the skin, the degeneration of the elastic tissue presents itself as yellowish plaques (hence the name) separated by grooves. The elements range in size from hempseed to pea, being in some cases confluent and forming stripes parallel to the lines of cleavage of the skin As a rule, the skin is loosely attached to the underlying structures and wrinkled The disease appears to be hereditary, running in certain families.

Microscopic examination of the skin reveals degeneration and thickening of the elastic fibres that are curled-up, fragmented and disintegrated They may be basophil and show calcification. The collagen fibres appear to be normal. Similar changes have been observed in the small arteries.

In most cases, the initial symptoms occur in the eyes, and the diagnosis is often made by an ophthalmologist, as the patients complain of visual impairment owing to hæmorrhages and effusions leading to atrophy. Occasionally, detachment of the retina is observed. The so-called "angioid streaks" presenting themselves in the ophthalmoscope as white glistening streaks along the vessels are due to degeneration of the elastic fibres in Bruch's membrane which may even burst. Degeneration of the elastic tissue in the blood vessels entails partly hæmorrhages and partly dilatation of the great vessels, e.g the aorta (Urbach & Wolfram, 1937).

Epidermolysis Bullosa Hereditaria

This disease, characterized by subepidermal vesicles and bullae, affects the skin and the oral mucosa. The dystrophic variety exhibits hæmorrhagic bullae leaving atrophic scars Generally, the changes involve the dorsal aspects of the joints, but vesicles have been observed in all sites The skin is extremely vulnerable, showing cyanosis and small hæmorrhages. In the connective tissue, elastic tissue is present in very small quantities or entirely absent. The latter applies mainly to the dystrophic variety—in diseased skin as well as in apparently healthy areas. All vessels are dilated and thin-

walled, exhibiting degeneration of the elastic tissue. The pathogenetic significance of this atrophy of elastic tissue has not been definitely elucidated.

STRIAE ATROPHICAE

These are first pink and later white atrophic lines in the skin due to bursting of the connective tissue. They have also been termed striae distensae, appearing in most cases where the skin is stretched, e.g. on the abdomen during pregnancy, on the breasts after lactation, in Cushing's syndrome mainly in sites that are stretched, but also on the upper arms, etc. In addition, they occur in very obese persons.

Striae atrophicae are caused by degeneration of the dermal fibres As mentioned on p 290, all elastic tissue, including that of the vessel walls, degenerates in hypercorticoidism In the early stages, there is fraying of the elastic fibres as well as slight swelling and distortion of the collagen bundles. In striae of long standing, the elastic tissue is usually completely lost

CUTIS HYPERELASTICA (Ehlers-Danlos' Syndrome)

The skin may be pulled in large, loose folds from the underlying tissue, snapping back owing to increased elasticity The skin is fragile, particularly over the joints "Pseudotumours" in the form of fibrosed hæmorrhages are encountered. Hyperflexibility of the joints lends the condition a systemic character

All mesenchymal structures show increased elastic tissue, the fibres being coarse and twisted The collagen bundles are more or less degenerated

Disorders Predominated by Changes in the Connective-Tissue Cells

URTICARIA PIGMENTOSA

This is a skin disease characterized by brown macules, infiltrates, or soft tumours When rubbed, the elements become urticarial

The outstanding microscopic feature besides melanin pigmentation of the basal epidermal cells is the more or less dense accumulation of mast cells in the corium, particularly its superficial layers In some cases, especially the nodular forms, the infiltrations are tumour-like (Fig. 7). The

systemic nature of the disease is evidenced by radiological signs of translucencies and irregularities in the bones in some cases (Sagher et al, 1952,



Fig. 7.
Urticaria pigmentosa Photomicrograph
Tumour-like accumulation of mast cells in the connective tissue

and others) Although skeletal biopsies have not yet been carried out, these findings are believed to be due to mast-cell infiltrations In urticaria pigmentosa an increased vascular fragility and cutaneous hæmorrhages may occur (Asboe-Hansen, 1950 b).

Fibromatosis

Fibromas are common tumours in the skin as well as all over the connective tissue system Certain conditions, such as acromegaly, predispose to fibromatous growths. The tumours are made up largely of fibroblasts and collagen bundles In some growths part of the cells have shown phagocytic properties, having ingested fat or hæmosiderin Where these cells predominate, the growths are named *histiocytomas*. A special variety is *Recklinghausen's disease,* which is characterised by multiple pigmented spots and soft or firm, small or large, pedunculated or sessile tumours in the skin or subcutaneous tissue. The growths are supposed to originate from the perineural connective tissue, but it has also been maintained that they arise from cells in Schwann's sheaths. The cells possess all the characteristics of fibroblasts; the collagen bundles are arranged in whorls. There is a more or less ample mucinous ground substance.

These growths may be encountered *inter alia* in the nervous system—central as well as peripheral—in other organs, and not infrequently in the bones (in a subperiosteal situation) The symptoms are due to their pressure on the surrounding tissues.

Comment

The systemic skin diseases including the lesions mentioned in this chapter link dermatology to internal medicine. The clinical pictures of atrophy and degeneration, of increase and change of the ground substance, of fibrosis, elastosis, vascular phenomena as well as of cell proliferation and migration should be evaluated from the mesenchymal point of view by the internist as well as the dermatologist

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MESENCHYMAL ASPECTS IN OPHTHALMOLOGY

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Introduction

RECENT YEARS have seen profitable progress within the field of connective tissue research, the concept of "diffuse collagen disease" has been introduced (Klemperer, Pollack & Baehr, 1942), and epoch-marking papers have been published on results of treatment with ACTH and cortisone. This development has, among other things, contributed towards a better pathogenetic understanding of various eye diseases, whose treatment and prognosis have become essentially improved thanks to ACTH and cortisone. The eye diseases in question are mainly of an inflammatory nature. Conjunctivitis, scleritis, and iridocyclitis are well-known as complications or initial signs of joint diseases, such as rheumatic fever, rheumatoid arthritis, *as well as of the mucocutaneous syndrome, viz. pluriorificial* erosive ectodermosis, Stevens-Johnson's syndrome, and Behçet's syndrome, whose clinical manifestations are universally known (Comroe, 1941, Edstrom & Österlind, 1948, Franceschetti, 1946, François, 1954, Sorsby & Gormaz, 1946, Godtfredsen, 1949, Savin, 1951, Nutt, 1951, Sury, 1952, Pickering, 1951, Hart, 1951, Wayne, 1951).

The results achieved from connective tissue research constitute a useful aid for the ophthalmologist seeking to clarify the numerous unsolved and obscure pathogenetic problems relating to the above eye diseases, whether they occur as *apparently monosymptomatic phenomena* or form part of a generalized syndrome. Within the last few years several valuable papers have been published in the ophthalmological literature on collagen diseases and the ophthalmological equivalents displayed by the connective tissue structure of the eye, which may even present very polymorphous pictures (Christensen, 1951, Hollenhorst & Henderson, 1951, Stillermann, 1951, Nutt, 1951, Vail, 1952, Kratka, 1953). The dramatic results obtained by

the ophthalmologists from treatment with ACTH and cortisone have given rise to a rich literature, mainly clinical, but also experimental These results have also attracted attention outside the narrow circle of ophthalmologists, in the first instance among connective tissue researchers.

The observed relationship between eye affections and collagen or mesenchymal diseases is the cause of the present brief review of eye signs and symptoms in association with mesenchymal diseases A knowledge of the physiopathology and delimitation of mesenchymal diseases is taken for granted and will therefore not be further discussed in this place. The clinical syndromes are mentioned fragmentarily, with emphasis on the features relevant to the present analysis For more detailed information reference is made to the current text-books and the quoted fairly comprehensive reviews The orbital mesenchymal affections, especially the problems of exophthalmos, are mentioned elsewhere

Eye Signs in Association with Mesenchymal Diseases

The mesenchymal diseases and types of eye signs of interest for the present investigation appear from Table 1, where the mesenchymal diseases are divided in three groups according to the cardinal signs, though many of the syndromes overlap each other symptomatologically

Joints and Eyes

Mesenchymal diseases with the signs and symptoms localized chiefly in joints and eyes are acute as well as chronic arthritis due to infection, i e rheumatic fever, simple urethral polyarthritis or Reiter's disease, and rheumatoid arthritis, especially Still's disease and ankylosing spondylitis or Strumpel-Bechterew's disease

Rheumatic fever is comparatively rarely associated with eye signs. These manifest themselves in 5 to 7 per cent of the cases as uni- or binocular phlyctenular conjunctivitis, episcleritis, scleritis, tenonitis, and iritis, the latter most often of a serous type The fact that eye signs are rarer now (Nutt, 1951, Savin, 1951) than previously suggests that rheumatic fever has changed both qualitatively and quantitatively thanks to the latest progress of treatment Phlyctenular conjunctivitis, episcleritis, and scleritis are morphologically analogous expressions of the same physiopathological process, only differing topographically. The histopathological structure of the phlyc-

TABLE 1

Ocular signs and symptoms in mesenchymal diseases

Mesenchymal diseases		Ocular signs and symptoms			
Main sympt from	Subgroup of mesenchymal diseases	Conjunctivitis Episcleritis	Iritis Uveitis	Sjögren Sicca Syndrome	Retinopathy Optic neuritis
Joints	Acute: Rheumatic fever	+	+		
	Acute: Reiter's syndrome	++	+		
	Chronic: Rheumatoid arthritis	+	+	++	
	Chronic: Still's disease		++		
	Chronic: Ankylosing spondylitis		+++		
Skin and mucous membranes	*Muco-cutaneous syndromes*				
	Ectodermosis erosiva plurif	++			
	Stevens-Johnson's syndrome	++			
	Erythema exsudativum multiforme	++			
	Bechet's syndrome	+	++		
	Sarcoidosis (Boeck-Schaumann)				
	Acute type Uveo-Parotid fever (Heerfordt)		++		
	Chronic type		+	(+)	
	Lupus erythematosus disseminatus (Libman-Sacks' disease)	+	+		++
	Dermatomyositis and Sclerodermia				++
Vascular system	Periarteritis nodosa or polyarteritis nodosa				++
	Arteritis temporalis (Horton's disease)				+++

tena is identical with that of Aschoff's nodules in the heart and skin. The *eye signs, like the main disease, have an acute character.*

In *Reiter's disease* the triad of polyarthritis, urethritis, and eye signs is complete in 70 per cent Articular signs and symptoms are present in 98 per cent, eye signs in 90 per cent, urethritis in 79 per cent, and some of the patients experience an initial dysentery-like gastro-enteritis (Paronen, 1948). The eye affections consist most often in acute or subacute, endogenous, binocular conjunctivitis with moderate or scant discharge from the conjunctiva. A small proportion of the patients (about 10 per cent) present keratitis, kerato-iritis, or iridocyclitis, of the serofibrinous acute or subacute type. Reiter's disease occurs, as we know, predominantly in males, but a few instances have been observed of the disease in females (Zewi, 1947). Treatment with ACTH and cortisone is very efficient.

In the cases of *chronic arthritis* due to infection the eye signs, like the main disease, have a chronic intermittent character, occasionally with acute exacerbations They comprise iritis, iridocyclitis, as well as the sicca syndrome.

Iritis and iridocyclitis are rare complications of the current progressive chronic rheumatoid arthritis localized in the limbs, but are more frequent phenomena in Still's disease (15–20 per cent) and ankylosing spondylitis (30–50 per cent). The percentage figures differ in the various statements, because the frequency depends on how long the main disease has persisted and on the extent to which ophthalmological special examination by slit lamp has been carried through, as several cases of iritis run such an indolent course that they may be overlooked In Vesterdal and Sury's (1950) large case material, comprising 102 children with Still's disease 21 per cent had iridocyclitis (submitted to ophthalmological special examination). The iridocyclitis attending *Still's disease* is binocular in four-fifths of the cases and often complicated by band-shaped corneal opacity. Eye signs may in a small number of cases be the first manifestation of Still's disease, which is twice as frequent among girls as among boys As iridocyclitis may run an indolent course, with a normally looking eye and no pain, and as the relatively young children do not complain of their eyes, the eye lesion may be overlooked and relevant treatment therefore omitted, with a chance of troublesome complications Patients with Still's disease ought accordingly always to be submitted to ophthalmological special examination even if subjective complaints are absent. ACTH and cortisone have proved very efficient against Still's disease and the ocular complications (Davis, 1953).

The iridocyclitis occurring in association with *ankylosing spondylitis* has a fibrinous acute or subacute character and may be the initial sign. In cases of iritis where the patient cannot advance the chin to the chin-support of the slit lamp, the ophthalmologist should suspect a diagnosis of ankylosing spondylitis If the disease has persisted for several years, from one-third to one-half of the patients will present intermittent iritis. The fact that males predominate among the patients with ankylosing spondylitis (nine-fold more males than females), unlike in other types of rheumatoid arthritis, has stimulated to renewed aetiological reflections In a monograph comprising 117 cases of ankylosing spondylitis Romanus (1953) claims, on the basis of very thorough clinical, radiographical, histological, and serological examinations, that nearly all patients with ankylosing spondylitis previously have had a genito-urinary infection, most

often prostate-vesiculitis, occasionally of the type of Reiter's disease, which also preferably affects males Future investigations must show whether Romanus' interesting observations hold good. If so, they open out new aetiological and pathogenetic fields

The sicca syndrome, or Sjogren's disease is a characteristic phenomenon in primary rheumatoid arthritis only, with a frequency of 10 per cent (*Stenstam, 1947*). The clinical picture, described in detail by Sjögren (1933, 1951), Henderson (1950) and S Holm (1949) and correlated with Plummer-Vinson's *syndrome and ariboflavinosis* (*Godtfredsen, 1947*), is a generalized systemic affection of the secretory structure in the upper respiratory and alimentary tracts, lacrimal glands, pancreas, and vaginal glands, with humoral changes (hyperglobulinaemia). The symptomatology is characterized by keratoconjunctivitis sicca · the epibulbar conjunctiva is stainable with Rose-Bengal's solution, and lacrimation is absent or reduced. Further, xerostomia, atrophic rhinitis, histamine-refractory achylia, and desquamatory colpitis are found. The eye affection is characterized primarily by atrophy of the lacrimal gland, giving rise to trophic disorders of the epithelium of cornea and conjunctiva. Treatment with ACTH and cortisone has been tried, but with no definite results (Sjogren & Eriksen, 1952). "Artificial tears" locally are used as a symptomatic treatment.

Skin and Mucous Membranes

Mesenchymal diseases with *cardinal signs and symptoms from the skin* and mucous membranes comprise in the first instance the mucocutaneous syndromes (*plurionficial erosive ectodermosis*, exudative erythema multiforme, Stevens-Johnson's syndrome, Beçhet's syndrome), and further sarcoidosis, disseminated lupus erythematosus, dermatomyositis, and scleroderma.

The mucocutaneous syndromes are characterized by affection of the mucosa of the natural bodily orifices, manifesting itself by different forms of aphthous, bullous, erosive ulcerous or pseudomembranous conjunctivitis, stomatitis, balanitis, vulvovaginitis, and proctitis. The term plurionficial erosive ectodermosis is used synonymously with Stevens-Johnson's syndrome, which represents a special course of exudative erythema *multiforme* (Ustvedt, 1948, Strom, 1948, Mattson & Carlberg, 1953, François, 1954). Besides a pseudomembraneous *conjunctivitis*, *an acute or subacute iridocyclitis* may be observed, which in Behçet's syndrome presents purulent

exudation with hypopyon Though the skin lesion in Behçet's syndrome often resembles erythema nodosum, there is an increasing tendency to include this syndrome, too, under exudative erythema multiforme. The aetiology of the mucocutaneous syndromes is often obscure. There is much evidence to suggest that the interrelated and overlapping syndromes may be released by one or more trigger mechanisms, both toxic, bacterial, and a virus The virus-aetiology is suggested in a recent study on Behçet's syndrome (Sezer, 1953) The mucocutaneous syndromes generally respond dramatically to ACTH and cortisone (Hauge, 1952)

Sarcoidosis or benign lymphogranulomatosis (*Boeck-Schaumann*) most often occurs in the chronic form, with sarcoids in the skin and mucous membranes, polyadenitis, osseous changes, and hyperglobulinaemia More rarely it manifests itself in the acute form of *uveoparotid fever* (*Heerfordt*). In Heerfordt's syndrome the uveitis appears as an acute serofibrinous iridocyclitis; there are concurrent or chronologically displaced febrile diseases, and very often cranial nerve palsies, especially facial nerve palsy, indicating the presence of leptomeningitis (Godtfredsen, 1945) In chronic sarcoidosis the eye signs are likewise chronic, occurring in the form of iridocyclitis, more often of a non-granulomatous than of a granulomatous type. Sarcoid granulomas may very occasionally be present in the lacrimal gland and the lacrimal sac

Disseminated lupus erythematosus is a severe general disease comprising fever, polyadenitis, weight loss and characteristic skin lesion, polyarthritis, polyserosynovitis, especially in the form of endocarditis, as well as pluriorificial mucosal affection analogous to that mentioned in connection with mucocutaneous syndromes The eye signs consist (Cordes & Aiken, 1947) in different forms of conjunctivitis and angiospastic retinopathy with cotton-wool patches, retinal haemorrhages, and oedema of the disc, in other words, a fundus picture simulating that in hypertensive retinopathy, but characteristically *without* arterial hypertension The ophthalmoscopic finding alone may therefore be a valuable aid in the diagnosis of an obscure general disease ACTH and cortisone allegedly have a favourable effect

Dermatomyositis and scleroderma are two rare diseases bearing a close clinical resemblance to disseminated lupus erythematosus. Dermatomyositis involves both the skin and the muscles, whereas scleroderma manifests itself by atrophic skin and fibrous sclerosing of the subcutaneous tissue The eye signs in dermatomyositis are episcleritis, scleritis, iritis, and exuda-

tive retinopathy, like in lupus erythematosus. Scleroderma may, unlike other mesenchymal diseases, be associated with cataract.

Vascular Lesions

The mesenchymal diseases where vascular disorders are the cardinal symptoms comprise periarteritis or polyarteritis nodosa and arteritis temporalis (Horton).

In *periarteritis nodosa* the generalized disseminated vascular affection is localized in the small arteries and arterioles, where the nodose changes of the vascular tissue check or completely arrest the blood flow, resulting in partial or total infarction in the areas supplied by the respective vessels. In addition to vascular changes, the syndrome comprises polyarthritis, polyadenitis, and splenomegaly, presenting, in other words, a polymorphous picture reminiscent of disseminated lupus erythematosus. The eye signs also simulate those seen in lupus erythematosus and dermatomyositis. Thus, they consist of angiospastic exudative retinopathy with partial or total occlusion of the retinal arterioles, resulting in cotton-wool patches, haemorrhages, retinal or subretinal oedema, which may cause retinal detachment. Nodules may occasionally be observed in the choroidal vessels. Here, too, ophthalmoscopy is therefore an important diagnostic aid. The disorders of the retinal vessels cause severe visual disturbances of the same type as in *arteritis temporalis*.

The term *arteritis temporalis* is a nosographically too restricted designation for the generalized vascular disease that has attracted increased attention within recent years, partly owing to the promising results of treatment with ACTH and cortisone, and partly because of the rather high incidence of severe eye signs and symptoms among these patients. A typical feature of the disease is the local affection of the temporal arteries, which become red, swollen, nodular, and stenosed. The general signs and symptoms of the disease are fever, malaise, headache, anorexia, weight loss, and leucocytosis. Women are more frequently affected than men, and the majority of the patients are between 55 and 70 years of age, suggesting that certain sclerotic vascular changes may be predisposing factors. Histopathologically the arteritis is localized in the intima and the media, and the histological picture shows points of resemblance to periarteritis nodosa and disseminated lupus erythematosus. Eye signs are present in about 40 per cent of the patients (Bruce, 1950, Parsons-Smith, 1952), in the forms

of ischaemic retrobulbar neuritis (in a scant two-fifths of the patients), occlusion of the central retinal artery (in a scant one-fifth), while the remaining vascular lesions occur in the fundus (in just over two-fifths), consisting of subretinal or retinal haemorrhages, periarteritis, arteriospasms, and phlebitis. These findings, differing morphologically, are physiopathologically analogous expressions of vascular insults on topographically different sections of the vascular supply to the optic nerve and the retina The eye affections result in permanent blindness of more than half of the affected eyes. 38 per cent of the patients become blind of both eyes A small proportion of the patients preserve visual field rests or a defective central vision The eye signs come on suddenly as typical vascular insults and may be initial signs of arteritis temporalis As there is an interval between the manifestations of the disease in the two eyes, cortisone treatment can be instituted within this interval The experiences gained from this treatment (Whitfield, Cooke, Jameson-Evans & Ruud, 1953) are, however, very limited. As it is a well-known fact that ACTH and cortisone have a thrombosis-stimulating effect, hormone treatment must be regarded as dangerous in cases of occlusive retinal diseases, of which a great number seem provoked by ACTH treatment

Discussion

It appears from this brief review that eye signs are important integral components of this often very polymorphous picture presented by mesenchymal diseases The eye signs may be among the first as well as among the most frequently occurring phenomena (conjunctivitis in Reiter's disease, iritis in Still's disease and ankylosing spondylitis, fundus changes in disseminated lupus erythematosus and arteritis temporalis). Ophthalmologists can therefore contribute essentially towards diagnosing mesenchymal diseases Conversely, all patients with a suspected or recognized mesenchymal disease ought to be referred to ophthalmological special examination, because indolent uveitis, iridocyclitis, and retinal lesions may otherwise be overlooked and relevant treatment omitted

The eye affections complicating mesenchymal diseases may be divided morphologically into two main groups

1) The inflammatory exudative affections localized in the external membranes of the eye (conjunctivitis, keratitis, scleritis, iritis).

2) The lesions due to disturbances of the vascular supply to the retina and the optic nerve (retinopathy, optic neuritis).

The inflammatory-exudative and the vascular eye lesions represent morphological equivalents of the mesenchymal disease concerned as regards nature and course Occlusive retinal lesions are seen in association with *arteritis temporalis*, but not iridocyclitis In cases of ankylosing spondylitis, on the other hand, we find iridocyclitis, but not retinopathy. The eye affection follows the main disease with regard to aetiology and pathogenesis The eye signs, like other manifestations of mesenchymal diseases, are released by one or more agents Their onset has an allergic-hyperergic character, the form of the mesenchymal tissue reactions concerned depending in some measure on the constitution of the host organism, as well as on hormone environment, hereditary conditions, physical and emotional stress, etc.

The various mesenchymal diseases present as common features two different physiopathological tissue changes, which may occur isolated or combined in different ways, thus characterizing the individual syndromes One change is a disintegrating fibrinoid degeneration with breakdown of the cells, fibrils, and amorphous matrix of the connective tissue, while the other consists in a restoring productive fibroblastic proliferation, including the reticulo-endothelial system The eyes of patients with mesenchymal disease, in fact, display the changes that fibrinoid degeneration and fibroblastic proliferation may effect in the connective tissue of the eye, considering the special anatomical conditions. Such changes are, for instance, phlyctene, nodose scleritis, fibrinous or granulomatous iridocyclitis, and exudative retinopathy The rare perforating scleromalacia is the extreme of fibrinoid degeneration (François, 1951, Hobbs, 1952).

A close clinical, morphological, chronological, pathogenetic, and physiopathological relationship thus exists between eye lesions and the respective mesenchymal diseases. This view has been borne out by treatment with ACTH and cortisone, the response to which may even be used as a criterion of the presence or not of a mesenchymal disease

Treatment with ACTH and Cortisone

The dramatic response to ACTH and cortisone of the inflammatory-exudative eye lesions complicating mesenchymal diseases justifies a brief

recapitulation of the views advanced as to how and why the eye affections are particularly susceptible objects for treatment. A further account of the use of ACTH and cortisone within ophthalmology is beyond the scope of the present paper. This has been thoroughly discussed in the literature (Woods, 1950, 1951, 1952, Purnell & Leopold, 1952, Duke-Elder, 1951, Thygeson, Hogan & Kimura, 1953, Vesterdal, 1951). The modes of action of ACTH and cortisone in eye diseases are not yet quite clear, but it is supposed to be due to a direct action of the adrenocortical hormones on the mesenchymal fraction of the inflamed tissue Woods (1952) concludes, on the basis of comprehensive experimental investigations into the effects of ACTH and cortisone, that "cortisone topically or parenterally and ACTH parenterally will (a) suppress the inflammation induced by the various recognized ocular hypersensitivity reactions, the ocular reactions due to toxins or irritants, and the inflammations due to bacterial infections, (b) will inhibit neo-vascularisation, (c) will, within limits, reduce fibroblastic activity in the stroma and the endothelial regeneration" These experimental experiences are on a line with those gained within human clinical medicine. The reason why such excellent results can be achieved with ACTH and cortisone appears from Wood's statement (1952) "when ocular inflammation is the result of acute trauma—allergic, toxic, or physical—the reaction of the tissues as usual is self-limited and the control of inflammation over the natural life of the tissue reaction may simulate a complete cure. ACTH and cortisone therefore find their highest usefulness in allergic reactions of the external eye and non-granulomatous inflammations of the uveal tract" By means of ACTH and cortisone the ophthalmologists can check the exudative phases of the disease and protect the picture-forming and perceptive structures of the eye against deleterious agents, until the cause of the disease has been eliminated or overcome by the organism's own immunobiological forces, aided by antibiotics etc The treatment of the various ocular affections complicating mesenchymal diseases coincides in part with that of the main disease, but in addition, supplementary local treatment may be desirable, with cortisone or hydrocortisone instilled, applied as ointment, or given as subconjunctival injections The treatment must be extended over a suitable length of time and cease by steps to avoid recurrence In some cases it may be necessary to supplement the treatment with ACTH and cortisone by pupil-dilating drugs, local heat on the eye, etc, dependent on the conditions in hand.

Summary and Conclusion

A clinical analysis has been made of eye lesions (conjunctivitis, episcleritis, scleritis, iritis, the sicca syndrome, and exudative angiospastic retinopathy) complicating mesenchymal diseases with cardinal signs and symptoms from

(a) joints rheumatic fever, Reiter's disease, rheumatoid arthritis,

(b) skin and mucous membranes. pluriorificial erosive ectodermosis, sarcoidosis, disseminated lupus erythematosus, dermatomyositis, and scleroderma,

(c) vessels periarteritis nodosa and arteritis temporalis

Eye signs are integral components of the polymorphous clinical pictures. They are frequent and generally occur early in the illness The ophthalmologist can contribute essentially towards diagnosing mesenchymal diseases. Conversely, patients with a suspected or recognized mesenchymal disease ought to be referred to ophthalmological special examination, in order that indolent eye lesions may be submitted to relevant treatment The eye affections represent morphological, pathogenetic, as well as physiopathological equivalents to the changes that in mesenchymal diseases occur in other parts of the body in the forms of fibrinoid degeneration and fibroblastic proliferation ACTH and cortisone are very efficient against the various eye lesions complicating mesenchymal diseases. As the eye signs represent an allergic-hyperergic tissue reaction, usually self-limited, the ophthalmologists, perhaps more than many other specialists, profit particularly by treatment with ACTH and cortisone, with which they can check exudative phases of the disease and thereby prevent the delicate structures of the eye from being exposed to deleterious agents

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FIBROSES DUE TO INJURY

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Introduction

WHEREVER TISSUE is exposed to injury, it responds by inflammation which is the local reaction of the body to irritation The inflammatory reaction presents two phases The object of the first is to destroy and remove the irritant, the object of the second is to repair the damage done to the tissues. The former is subserved by the wandering mesodermal cells, whether of the blood or the tissues, the latter by the fixed cells The further course of the inflammation depends chiefly on the nature and intensity of the irritant, the tissue affected, and the duration and nature of the irritation In cases of acute, mild injury, the inflammation seldom leaves any traces in the tissue Following more severe injury, involving extensive destruction of cells and necrosis which will act as a constant irritation after the actual injury has ceased or in cases of persistence of the original injury—e g chronic infections or foreign bodies—the evidence of an acute reaction, particularly the hyperæmia, will disappear The cells of acute inflammation are replaced by cells of a more chronic type, primarily lymphocytes The most significant feature of this chronic inflammation is a proliferation of fibroblasts and a gradually increasing production of fibrous tissue

The processes that take place at the traumatized site are in broad features as follows The irritation gives rise to the formation of an effusion containing fibrinogen and sometimes mixed with blood This effusion *coagulates and deposits fibrin in which* there will be formation of ground substance and fibrils by invading cells, and ingrowth of vessels and nerves —in other words connective tissue is formed. In cases of continued irritation, mast cells and fibroblasts proliferate and the resulting fibrous tissue may aquire the character of cellular fibrous tissue or else the fibroblasts may be stimulated to lay down collagen fibrils rather than to multiply, with the result that dense acellular connective tissue is formed.

The irritation proper may be of widely different nature, and the clinical signs of the fibroses depend entirely on the location This paper is devoted to the conditions that may occur on the basis of fibroses provoked by injury.

Skin and Subcutaneous Tissue

Fibrous scars are common phenomena that may follow upon all kinds of wounds, burns, contusions, incised wounds, etc. The degree of fibrosis depends on the severity of simultaneous irritation during healing, viz. infection and foreign bodies, including necrotic tissue and suture material In addition, fibrosis is more apt to occur where there is traction on a wound. In brief, any condition that delays healing promotes the development of fibrosis Possibly, nutrition, anæmia, and age also play their parts (Levenson, Birkhill & Watermann, 1950) Extensive burns and contaminated wounds frequently give rise to fibroses, which seldom follow upon clean, smooth operative wounds. In some cases, keloids form They are due to excessive formation of scar tissue. A keloid manifests itself as a large, thick, elevated tumour-like mass in the skin, and it sends out claw-like extensions as it grows It is particularly common in scars following burns. Individual differences are marked, some people developing hyperplasia in all, even small and uncomplicated wounds Keloid is said to be particularly common in negroes.

The great majority of fibrous scars do not give rise to subjective complaints apart from the cosmetic and mechanical aspects, but in some cases they may be painful, probably owing to the pressure of the fibrous tissue upon the nerves Fibrous scars have a marked tendency to shrink. If they are localized over joints, they limit the joint movements, desmogenous contractures occur, and if the condition is allowed to persist, the result may be irreversible changes in the joints, subluxation and osteoarthritis, in some cases even ankylosis Where fibrous scars are situated at the natural orifices of the body, the shrinkage may of course give rise to various complaints, such as e g difficulty of eating, difficulty of defæcation and urination, and in the eyes ectropion, epiphora, and consequent conjunctivitis and keratitis.

A fairly common syndrome that has hitherto received little attention, so-called *adiposalgia*, is also due to fibrosis of the subcutaneous adipose tissue. This condition occurs mainly in obese females; it may occur at all

ages, but usually around 50 years. It manifests itself by pain and tenderness of the adipose tissue, often on the legs, but it may also occur in other sites, not infrequently at the elbow. The pain is of a constant, dull, aching character and occasionally very severe paroxysms of pain occur. At times, the pain is projected deeper, to the knee and ankle joints which may feel stiff. Objective examination shows characteristic, firm, tender nodules in the adipose tissue. This condition must be distinguished from thrombophlebitis. It has been described in detail by Stockmann (1911) and has since that time been mentioned by a few authors (Seifert, 1951, Schmitt & Pichler, 1951, Schwarzweller, 1952). Microscopic examination shows irregularly spread, patchy, chronic inflammation of the subcutaneous connective tissue. The fibrous tissue in such a patch is thicker and denser than normal, with numerous fibroblasts; the small nerves running through it are in a condition of interstitial inflammation and the small blood vessels show periarteritis and endarteritis The intervening adipose tissue may be completely normal This is a typical reaction to chronic local irritation, and the condition is probably due to toxins or bacteria of various kinds It has been reported by Stockmann in acute rheumatism, influenza, mucous colitis, etc At times, however, no definite cause can be found, and possibly hormonal factors play a role (Schmitt & Pichler, 1951)

Muscles, Fascia, Joint Capsules, etc.

Fibrosis that goes deeper, may involve various other structures and compromise their function. When the tissue sustains an injury, there will as in other sites be serofibrinous effusion followed by deposition of fibrin between the various tissue layers, around the joints, tendons, tendon sheaths, ligaments, and within and between the muscles. In this fibrin connective tissue will form and again shrink so that all these structures will be plastered together with firm adhesions The nature of the primary injury differs Crushing of soft parts, fractures, distortions, infections, hæmatomas, or ordinary incised or operative wounds leading to immobilization. Oedema and tissue fluids are not removed by the muscular pump, and cicatrization rapidly entails limitation of movement The so-called *post-traumatic stiffness of the hand* is a typical and important example which deserves particular attention. Such a hand presents an extremely characteristic appearance, being stiff, smooth, atrophic, with tapering fingers, and fre-

quently slight cyanosis The wrist presents a flexion of 180° or slight volar flexion, the metacarpo-phalangeal joints dorsal subluxation owing to shrinkage of the lateral ligaments, and the proximal and distal inter-phalangeal joints are semi-flexed The condition is often complicated by causalgia making the hand painful and hypersensitive, so it is extremely incapacitating

Another lesion of similar nature is *humero-scapular periarthritis* in which case the fibrosis often is localized around the subscapular tendon and in the subacromial bursa. In *torticollis*, there is marked fibrosis of the sternocleidomastoid muscle, probably a result of bleeding in the muscle caused by birth injury This tense muscle acts as a backstay, pulling the head towards the affected site It may cause scoliosis of the cervical column and facial asymmetry

The *trigger phenomenon* or sudden snapping movement of a finger may arise wherever tendons slide over each other, through tendon sheaths, or under ligaments It often affects the flexor tendons of the long and ring fingers, being due to trauma, either single or multiple, usually incurred in grasping The ligamentous sheath and flexor tendon are pinched be-tween the object and the head of the metacarpal until local tenosynovitis and consequent fibrous thickening and constriction is formed in the tendon sheath and a local swelling in the tendon (Bunnell, 1948)

In *poliomyelitis*, fibroses and contractures of the fascia and paretic muscles are very common, particularly in the tensor fasciae latae and in the fascia plantaris In fact, the fibroses may occur at all sites and give rise to severe contractures In this condition, the fibroses are possibly merely a result of immobilization, but they occur with striking rapidity compared with other immobilizing diseases On the other hand, the extent and severity of the fibroses does not appear to be proportional to the severity of the paralysis, but this is a field that remains to be elucidated.

In *muscular rheumatism* there are often firm nodules and infiltrations in the muscles, so that it would be reasonable to presume the presence of fibroses These lesions have, however, been examined microscopically by numerous workers and all have failed to demonstrate fibrous changes. Wallroff (1951) found degenerative changes of hyaline nature.

A disease which previously was interpreted as a traumatic lesion is *Dupuytren's contracture* (Dupuytren, 1834, Niederland, 1932, Schröder, 1934) This is fibrous thickening and shrinking of the palmar fascia with resulting flexion contracture of the fingers, most frequently the ring and

little fingers, but the lesion may affect any finger. Microscopic examination shows infiltration, and in more advanced stages only firm scar tissue (Meierding, Black & Broders, 1941). The patients do not infrequently present similar changes in the plantar fascia and in the septum separating the corpora cavernosa and tunica albuginea of the penis—so-called *Peyronie's disease* (Korhonen, 1950, Busford, 1952) and occasionally in other fasciae and tendon sheaths Skog (1948) found Dupuytren's contracture to be more common in epileptics (42 per cent) than in normal subjects (2 per cent) The disease is a typical hereditary lesion. In addition to the hereditary nature of the disease and its frequent association with other similar conditions, the arguments against the causal role of trauma are as follows: It is usually bilateral although the right hand is most exposed to trauma Palmar injuries are not, however, followed by Dupuytren's contracture more often than may be ascribed to chance coincidence. Studies of large groups of the population have shown the lesion to be more prevalent in people who do not perform hard manual work than in manual workers in the proportion 55 to 45 respectively (Bunnell, 1948). In Teleky's (1939) opinion, however, the disease is due to an interplay of predisposition and exogenous causes. He examined a special group of workmen whose work involved a constantly repeated, sudden stretching of the palmar fascia. This group showed Dupuytren's contracture in 17 per cent, whereas it occurs in only 1 per cent of persons with light or no manual work. The scars left by operation for these lesions often develop fibrosis, possibly because the changes often reach as far as the dermal connective tissue, so that the operation cannot become radical

Pseudarthroses also present a kind of traumatic fibrosis Following fracture, healing and bone formation may be delayed for some reason or other, e g deficient immobilization etc. The bone ends become smooth, the bone cavities close, and between the two bone ends a thick layer of firm fibrous tissue is formed that often gives rise to pain and always to instability.

Also within obstetrics, traumatic fibroses are of great importance Hess, in 1953, showed that healing of the uterus following Caesarean section takes place mainly by the formation of fibrosis. Such scars contain almost exclusively firm fibrous tissue and only occasional muscle fibres. He states that rupture of the uterus, occurring in 1 out of 2000 normal persons, takes place in about 1 out of 70 persons with a history of Caesarean section.

Serous Membranes

Peritoneal adhesions constitute a common and often serious disease. Their development is completely analogous with other posttraumatic fibroses, and the primary factor is the damage to the serosa that may be due to a number of different injuries, of mechanical, chemical, thermal nature, foreign bodies etc (Boys, 1942, Zachariae, 1954). In this connection it is worth mentioning that talc induces adhesions. Zachariae, in 1954, after sprinkling the small intestine and mesentery of rabbits with 250 mg. of sterile talc, found in 100 per cent dense adhesions that completely plastered the intestines together In this form of traumatic fibroses there is a special factor to be considered, i e that the organs are glued together and thus disturbed in their function Various uncharacteristic painful conditions may occur owing to traction on the peritoneum by the adhesions, and in serious cases intestinal obstruction may arise owing to a kink or clamping off of the bowels Duff (1939) analysing the frequency of peritoneal adhesions at autopsy, found such adhesions in 30 per cent of persons who had not been submitted to laparotomy and in 90 per cent of those who had a history of laparotomy Payr (1914) states that 3.5 per cent of all laparotomies are performed for intestinal obstruction, and according to Cristopher 30–40 per cent out of this number are due to peritoneal adhesions

Pleural adhesions are in most cases caused by infection They are of particular significance in tuberculosis, as they may prevent the collapse of the lung in artificial pneumothorax

In chronic *pericarditis*, that is most frequently associated with tuberculosis, rheumatic fever, and Pick's disease, but which may also be a sequel to certain types of acute pericarditis, for example that accompanying septic states, the heart may be compressed by a progressive fibrosis of the pericardium with resulting signs of congestive failure This type of pericarditis is constrictive There is also a chronic form of *non-constrictive* pericarditis that may give rise to symptoms, because the cardiac function is inhibited by fibrous adhesions to the adjacent mediastinal structures and the chest wall (Bost, 1939)

Nervous System

As is well-known, nerve cells possess very little capacity to regenerate—in the central nervous system none at all In other words, destroyed nerve

cells are replaced by glia, also called "the connective tissue of the brain". This differs, however, from connective tissue in other sites in being derived mainly—at least the astrocytes and oligodendrocytes—from the ectoderm, only the microglia being probably of mesodermal derivation. According to Rio-Hortega, (Maximow, 1941) microglia originates from mesodermal cells of the pia mater which migrate into the central nervous system along the blood vessels.

Traumatic destruction of the nerve cells leaves a scar that gives rise to symptoms varying according to its location. In the peripheral nervous system, there will be pareses and disturbances of sensibility, but regeneration may occur In the central nervous system, such scars need not give rise to symptoms if they are situated in the so-called silent zones, but cicatrization in the motor zone results in Jackson's epilepsy (Krabbe, 1945), manifesting itself as seizures starting in the peripheral areas and spreading centralwards A similar syndrome, but of a sensitive nature, may occur if the scar affects a sensory zone. Infections or hæmorrhages in the membranes of the central nervous system may be followed by adhesions plastering them together, and giving rise to symptoms by influencing the flow of the cerebrospinal fluid (Krabbe, 1945).

Lungs

Quite special factors apply to traumatic fibroses of parenchymatous organs. Owing to the injury, part of the specific cells perish and are replaced by fibrous tissue, so that their function is lost. Furthermore, otherwise normal cells in the surroundings may be disturbed in their activities by pressure or traction exerted by the fibrous tissue. Similarly, the blood and nerve supply may be compromised, disturbing the activities of the specific cells

One of the most important varieties of traumatic fibrosis is *silicosis.* This is a disease of the lungs caused by the continuous inhalation of microscopic particles of free silica dust It is a common occupational disease, especially in mining districts. The report of the National Silicosis Conference (Frere, 1949) estimates that approximately two per cent of the working population of the United States or about one million workers, are exposed in some way to the potential hazard of silicosis

The inhaled silica particles are phagocytized and transported to the

lymph nodes where owing to chronic irritation they form fibrous nodules. The tracheobronchial lymph nodes are affected first, followed by fibrosis along peribronchial and perivascular lymph channels. These fibrous nodules and strands spread, become confluent, destroying the pulmonary tissue, so that in the terminal stage the appearance is one of massive fibrosis with extensive pulmonary consolidation The lesions are most pronounced at the apices, equally marked in both lungs In the basal parts there is invariably compensatory emphysema The symptoms are dyspnoea, pain, cough, and a debilitated general condition In addition, the resistance to tuberculosis is reduced Gardner (1934) says that at least 75 per cent of those in whom silicosis develops die of tuberculosis. Similar pulmonary diseases may be caused by asbestos, beryllium, and anthracosilica dust (De Laureal, 1949).

Acute diffuse interstitial fibrosis of the lungs is a disease characterized by sudden onset of cough, hæmoptysis, dyspnoea, cyanosis, and fever. Death from cardiac failure or pulmonary asphyxia has occurred in the reported cases after an illness of a few weeks The disease was first described by Hamman & Rich in 1949 The detailed description of the pathologic anatomy that these authors presented established the fundamental changes as diffuse proliferation of fibrous connective tissue which involved the interstitial tissue of the lungs, with subsequent alterations in the pulmonary circulation The alveolar walls are thickened, in the early stages of the process crowded with fibroblasts, and later mature scar tissue develops It is noteworthy that the alveoli themselves contain little or no cellular inflammatory exudate in contrast with what is seen in pneumonia. The ætiology is unknown At any rate, the lesion is not of bacterial nature, but probably due to virus or a chemical irritant (Hamman & Rich, 1944); possibly the underlying cause is obstruction of the lymphatic vessels (Callahan, Sutherland & Fulton, 1952).

Kidneys

When renal tissue is destroyed it is—like other tissues—replaced by fibrous tissue. Owing to the great reserve powers of the kidneys, however, large portions of the renal parenchyma can be dispensed with. Therefore, localized fibroses, e g. following infarct, hæmatoma, or localized infection usually fail to give rise to symptoms If, on the other hand, the action is

generalized—infectious, toxic, or vascular—and sufficiently prolonged, the destruction of renal tissue and its replacement by fibrosis becomes so extensive as to result in renal insufficiency. This is what happens in chronic glomerulonephritis the advanced stages of which show diffuse increase of interstitial tissue, while in chronic pyelonephritis there is characteristic periglomerular fibrosis

Page (1939) induced renal hypertension in experimental animals by wrapping one or both kidneys in cellophane, leaving the hilar structures free This gave rise to severe perinephritis and the kidneys became enclosed within a fibrocollagenous hull, 3–4 mm. thick The detailed mechanism is still obscure. Apparently it is not due to actual ischæmia, but possibly to inhibited pulsation of the kidney because it is so tightly encased (Corcoran, 1948). In man, hypertension may also be due in certain cases to perirenal fibrosis (Page, 1939).

Liver

In most instances, hepatic *cirrhosis* is due to slow, long-continued necrosis of the hepatic cells and their replacement by fibrous tissue. The cause of their gradual destruction is uncertain, but is no doubt often due to toxins of various kinds For instance, cirrhosis can be induced experimentally in the rat by means of carbon tetrachloride (Aterman & Ahmad, 1953). A cirrhosed liver presents a characteristic lobular appearance due to necrosis of the hepatic cells surrounding the central vein and their replacement by fibrous tissue, while the specific cells in the peripheral areas react by lively regeneration. Microscopic examination shows areas of varying size composed of liver cells and separated by broad strands of fibrous tissue containing many bile ducts It must be mentioned that there is no true lobular arrangement, the normal relation of the central vein being lost. The remaining liver cells usually exhibit fatty necrosis This is particularly common in cases of alcoholic cirrhosis. Davis (1949), in a series of liver biopsies from patients with cirrhosis, found the prognosis to be most serious where the formation of fibrous tissue was most active.

Endocrine Organs

This is not the place to enter into details regarding traumatic fibroses in the endocrine glands Suffice it to mention that in these sites they ac-

quire quite particular significance, disturbing the hormonal secretion and consequently exerting a profound influence on the entire organism

Foreign Bodies

The traumatism caused by the presence of foreign bodies invariably gives rise to fibrosis which is the defence of the organism towards the foreign substance. The fibrosis, however, differs in degree according to the nature of the substance, and a search for physical properties common to fibrosive materials has revealed a direct relationship between the fibrosive properties of a substance and its molecular structure (Evans & Zeit, 1948). Those substances which exist in a state of molecular asymmetry compatible with piezoelectric reactions are observed to produce fibrosis. Piezoelectricity is a mechanism whereby mechanical and electrized energy states may be converted from one to the other in either direction

Hormonal Influence

Since fibrosis may be interpreted as a further development of the normal fibroplasia that occurs in any formation of granulation tissue, e g in wound healing, it is to be expected that on the whole, hormonal influence on the formation of granulation tissue applies also to the development of fibrosis The effect of the various hormones will be briefly described below with special emphasis on the behaviour of the fibroblasts which seem to be the predominant cellular element in fibroses The hormonal actions are partly inhibitory and partly stimulating, the fundamental mechanism being widely different Taubenhaus & Amromin (1949), among others, have reported experiments showing the effect of hormones upon granulations induced by turpentine abscesses in the rat In hypophysectomized rats, the wall of the abscess is thin, the fibroblasts small and flat, collagen sparse, and in occasional coarse clumps Upon daily administration of the growth hormone, the fibroblastic response is of a different character. The wall of granulation tissue is thicker, the individual fibroblasts are moderately large and of a bipolar and multipolar variety The nuclei are moderately deep staining and the nucleoli prominent, the collagen is diffuse, abundant, and of a homogeneous character In normal animals, the growth hormone has a similar, but less promoting effect Desoxycorticosterone (DOCA)

promotes the process very much indeed, the granulation layer being very thick with extreme fibroblastic response. The fibroblasts are large with very large nucleoli and abundant cytoplasm with numerous cytoplasmic *processes, giving them a stellate appearance.* The collagen is very abundant and diffuse and of a glassy structure; fibrillogenesis is scanty. DOCA has no effect except when administered for a long time before the stimulus *and during the development of granulations as well Its effect is systemic,* and *in vitro* it is inhibitory. Cortisone prevents the promoting effect of DOCA.

Administration *of thyroxine to hypophysectomized animals increases* the fibroblastic response The fibroblasts are moderately large, the majority spindle shaped and others multipolar. The collagen is moderate in amount, pale and fibrillar. Thyroidectomized *animals form granulation tissue* it is true, but to a lesser extent than normal animals; the ground substance is altered, containing large quantities of mucopolysaccharide In normal animals, thyroxine has no influence on connective-tissue formation—in particular it cannot abolish the inhibitory effect of cortisone on granulation tissue

On administration of sex hormones—testosterone propionate and oestradiol propionate—the abscesses are very poorly defined, their walls thin, almost completely void of granulation tissue. The fibroblasts are sparse, very thin and spindle-shaped, and collagen appears in extremely rare, coarse clumps

Cortisone and ACTH are the most important inhibitory hormones, and in principle their effect is the same Granulation tissue becomes more sparse, the fibroblasts small, scanty and round, vascular ingrowth is decreased, and the ground substance subsides Cortisone exerts a marked topical effect unlike ACTH whose action is transmitted by way of the adrenals. In tissue cultures Cornmann (1951) found an elective, inhibitory effect of cortisone on fibroblasts, whereas the experiments of Alrich (1952) revealed no directly inhibitory effect on the fibroblasts

Treatment

The most obvious way is of course to prevent the occurrence of fibrosis. Mechanical traumatization during operations may be reduced, infections can be *fought* by *antibiotics,* and toxic actions avoided as far as at all



Fibrosis following operation for hæmangioma

Figs 1 and 2
Restricted flexion and extension 2 months after the operation

Figs 3 and 4
Movements after four injections of hydrocortisone topically (once a week)

possible An existing fibrosis formerly presented a fairly insoluble problem In some cases, surgical excision could be employed, but was often followed by recurrence In addition, a number of specific methods have been suggested in the treatment of certain traumatic fibroses, e g inhalation of aluminium dust in silicosis (Frere, 1949), vitamin E in Dupuytren's contracture, humeroscapular periarthritis, and Peyronies disease (Steinberg, 1951) X-rays have also been used a good deal in the treatment of the latter lesions and of keloids (Nikolawski, 1952)

In 1949 Ragan and associates noted delayed wound healing in patients receiving ACTH and cortisone and showed the inhibitory effect of cortisone on the formation of granulation tissue in animal experiments Thereafter, cortisone was introduced in the treatment of fibroses. It sounds incredible that an existing, firm, fibrous tissue should respond, and therefore cortisone treatment has often been combined with excision of the fibrosis (de Kleine, 1952) That cortisone does indeed affect existing fibrous tissue was shown in clinical practice by Baxter (1952), who treated postoperative fibrosis following operation for Dupuytren's contracture In his studies on cirrhosis of the liver, Morrione (1951) found that connective tissue can regress, the total hepatic collagen in livers rendered cirrhotic by

carbon tetrachloride regressing distinctly, when the stimulus is withdrawn. Cavallero et al (1951) and others have demonstrated that cortisone reduces cirrhosis induced by carbon tetrachloride, inhibiting particularly fibrosis, reticulosis, and parenchymatous regeneration without affecting the fatty degeneration.

For some years, cortisone has been used in the treatment of fibroses. Hydrocortisone has an effect that is in principle identical with that of cortisone (Conn, Louis & Fajans, 1951), but it is even more powerful and slightly soluble Upon topical injection it accumulates in a depot whence it exerts its effect (Figs. 1, 2, 3, 4).

Hydrocortisone acetate has already been used in the treatment of a number of lesions. Zachariae (1954) studied its inhibitory effect on the development of peritoneal adhesions, and Zachariae & Zachariae (1954) found unmistakable regression of fibroses that had followed upon injuries of the hand

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